# Spectroscopic analysis of protein Fe–NO complexes

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## Abstract

**The toxic free radical NO (nitric oxide) has diverse biological roles in eukaryotes and bacteria, being involved in signalling, vasodilation, blood clotting and immunity, and as an intermediate in microbial denitrification. The predominant biological mechanism of detecting NO is through the formation of iron nitrosyl complexes, although this is a deleterious process for other iron-containing enzymes. We have previously applied techniques such as UV–visible and EPR spectroscopy to the analysis of protein Fe–NO complex formation in order to study how NO controls the activity of the bacterial transcriptional regulators NorR and NsrR. These studies have analysed NO-dependent biological activity both *in vitro* and *in vivo* using diverse biochemical, molecular and spectroscopic methods. Recently, we have applied ultrafast 2D-IR (two-dimensional IR) spectroscopy to the analysis of NO–protein interactions using Mb (myoglobin) and Cc (cytochrome *c*) as model haem proteins. The ultrafast fluctuations of Cc and Mb show marked differences, indicating altered flexibility of the haem pockets. We have extended this analysis to bacterial catalase enzymes that are known to play a role in the nitrosative stress response by detoxifying peroxynitrite. The first 2D-IR analysis of haem nitrosylation and perspectives for the future are discussed.**

## Introduction

NO (nitric oxide) plays myriad roles in biological systems, including signalling and immune responses as well as being a key intermediate in denitrification [1,2]. Proteins containing transition metals are a particular target of NO, since its unpaired electron can interact and bond with the d-orbitals of these important cofactors [2]. Sub-toxic levels of NO are common in biology, such as during the NO-mediated signalling event induced by soluble guanylate cyclase [3]. However, high levels of NO have a wide variety of deleterious effects, leading to a situation known as nitrosative stress. This is further complicated by the high reactivity of NO and the inevitable inter-reaction with reactive oxygen intermediates leading to the production of secondary RNS (reactive nitrogen species) such as peroxynitrite. Theoretically, any protein containing a transition-metal cofactor is a target of nitrosative stress. Well-known targets include components of the respiratory pathway such as Cc (cytochrome *c*) oxidase and key enzymes of the tricarboxylic acid cycle such as fumarase and aconitase [4,5]. High levels of NO are known to inhibit the activity of Cc oxidase in mammalian systems. This effect is exacerbated in mice that cannot produce the haem-containing Mb (myoglobin) protein, suggesting a role for Mb as a sink for excess NO [5]. The role of NO as a signalling molecule in soluble guanylate cyclase-mediated control of vasodilation is another good example of the importance of Fe–NO complexes in haem proteins [3]. Because of the deleterious effects of excess NO on cellular function, most organisms have evolved a variety of mechanisms to detect and detoxify NO as well as repairing the damage caused to the metalloproteins that are required for cell survival.

Bacteria are exposed to NO from both endogenous and exogenous sources. NO is a key intermediate in bacterial respiratory denitrification, indeed, *Escherichia coli* and *Salmonella* both produce NO during anaerobic respiration [6]. These closely related organisms can be considered to have truncated respiratory denitrification pathways, since they lack a dedicated respiratory NO reductase such as that found in *Pseudomonas aeruginosa* [7]. Endogenously produced NO is instead detoxified by the flavorubredoxin NorVW and the flavohaemoglobin Hmp which convert NO into $N_2O$ and $NO_3^-$, respectively [6]. Pathogenic bacteria encounter exogenous NO during the infection process when the invading bacteria are internalized by cells of the innate immune system such as macrophages [8]. These immune cells induce the activity of the inducible NO synthase enzyme in response to PAMPS (pathogen-associated molecular patterns) such as flagellin and LPSs (lipopolysaccharides). Regardless of the source of NO, it is detoxified by NorV and Hmp with a contribution from the penta-haem respiratory nitrite reductase NrfA [6]. The NO-dependent gene expression of the *hmp* and *norV*

**Key words:** cytochrome *c*, electron paramagnetic resonance (EPR), myoglobin, NorR, NsrR, ultrafast two-dimensional IR spectroscopy.

**Abbreviations used:** AAA+, ATPase associated with various cellular activities; Cc, cytochrome *c*; DNIC, dinitrosyl iron complex; FTIR, Fourier-transform IR; GAF, cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *Escherichia coli* FhlA; 1D-IR, one-dimensional IR; 2D-IR, two-dimensional IR; Mb, myoglobin; MCD, magnetic CD; LMCT, ligand–metal charge transfer.

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genes in *E. coli* is controlled by the dedicated NO-sensing transcriptional regulators NsrR and NorR respectively [9,10]. These regulators are highly conserved in diverse bacterial lineages and are usually associated with NO detoxification systems [7,11,12]. Although NsrR and NorR control gene expression by very different mechanisms, they share the requirement of an iron cofactor for the detection of NO. NorR senses NO via a ferrous non-haem iron-containing GAF (cGMP-specific and -stimulated phosphodiesterases, *Anabaena* adenylate cyclases and *E. coli* FhlA) domain, while NsrR proteins use Fe–S clusters to detect NO [13–16]. Haem centres are also used in bacterial NO sensors such as *Ps. aeruginosa* Dnr and the *Shewanella oneidensis* and *Vibrio fischeri* H-NOX proteins [17–19]. Bacterial NO sensors and iron-containing transcription factors have been thoroughly reviewed elsewhere [20,21]. As such, this review will focus on the spectroscopic methods used to identify and characterize iron-based NO-sensing mechanisms and the effects of NO binding on protein structure and dynamics. Particular emphasis will be given to examples of the use of EPR and 2D-IR (two-dimensional IR) spectroscopy.

## Spectroscopic methods commonly employed to study protein Fe–NO complexes

UV–visible absorption spectroscopy is perhaps the most widely available spectroscopic technique for the analysis of iron-containing metalloproteins. Fe–S and haem cofactors are the most common form of iron moiety in proteins, and both are generally highly coloured, leading to absorption of energy in the visible light wavelengths. The classic example of this is the Soret peak observed approximately 405 nm in UV–visible analysis of haem proteins such as Mb and catalase [22]. UV–visible is widely used for the determination of the redox state of haem proteins, for example, reduction of Cc shows new spectral bands growing in at approximately 520 and 560 nm and loss of intensity at approximately 540 nm, and UV–visible absorption bands can also be used to detect binding of ligands such as NO.

EPR remains one of the most powerful techniques for the detection of Fe–NO complexes in proteins and whole cultures or tissue. EPR is a powerful tool for detecting unpaired electrons and as such has been extensively applied to the analysis of NO and other radicals. Unpaired electrons produce a magnetic field because they are spinning negative charges. The application of an external magnetic field to the sample leads to a change in the energy state of the unpaired electron, which can be measured as the absorbance of the externally applied energy by the sample [23]. EPR has been used to detect nitrosylation of a variety of NO-sensing cofactors, including Fe–S clusters, haem and non-haem iron centres [13,14,24,25].

IR absorption spectroscopy has been used for many years to investigate proteins. Primarily this has been through the sensitivity of the amide I (C=O stretching vibration) absorption frequency of the peptide bond to secondary structure and local hydrogen-bonding environment [26]. IR absorption experiments are mainly carried out via FTIR (Fourier-transform IR) spectroscopy methods and, while this is a useful tool, it lacks the time resolution required to probe the dynamic processes that occur in proteins. The latter include rapid conformational fluctuations of the secondary structure and intermolecular interactions involved in binding ligands that are often co-ordinated by hydrogen bonds. The latter typically have a lifetime in aqueous solution of approximately 1 ps ($10^{-12}$ s). Despite these constraints, FTIR has nonetheless been demonstrated to be a valuable method for the analysis of Fe–NO complexes [27,28]. The emergence of 2D-IR spectroscopy as a tool for the analysis of Fe–NO complex formation and the resulting structural rearrangements in NO sensors will provide valuable insights into these important proteins because it can provide time resolution of the order of 100 fs (see below). Other time-resolved techniques have been used to investigate NO binding to haem proteins, including time-resolved resonance Raman spectroscopy [29], which is sensitive to low-frequency vibrational modes involving the haem group and time-resolved pump–probe spectroscopies that have been used to observe ligand-rebinding dynamics of NO following photolysis [29–33]. Raman and resonance Raman spectroscopies are complementary IR vibrational techniques where the resonance condition uses lasers that tune into a molecular chromophore of interest. This yields an increase of several orders of magnitude in selectivity and sensitivity in comparison with standard Raman techniques. Raman spectroscopy has the advantage that it easily accesses the lower-frequency region of the IR and can be applied in water, in contrast with IR absorption measurements, which are generally made in $^2H_2O$. Combination of resonance Raman and IR techniques allows correlation of the $\upsilon$Fe–NO-stretching vibration (typically approximately 520 $cm^{-1}$) with environmental influences that perturb the strengths of the metal–NO bond. Resonance Raman has been used to investigate the Fe–NO structure in the distal pocket of mutants of Mb [34]. Resonance Raman has also been a valuable tool for the identification of (Fe–S) cluster types, including the identification of the (4Fe–4S) cluster in *Bacillus subtilis* NsrR [15]. Time-resolved Raman methods have been applied to the analysis of Fe–NO complexes such as the ultrafast structural transitions of haem centres induced by NO binding [29].

## Spectroscopic analysis of the NorR NO-sensing mechanism

Genetics provided the first evidence that NorR is a NO-dependent transcriptional regulator responsible for the expression of the NorV flavorubredoxin in *E. coli* [9]. The importance of NorV in the nitrosative stress response was further indicated by the fact that *norV* is the most highly up-regulated gene in NO-treated cultures of *E. coli* under both aerobic and anaerobic conditions [35]. Since NO interacts

with the d-orbitals of transition metals and considering the relative abundance of iron-based protein systems, it was hypothesized that NorR detected NO in this manner. The first direct evidence of this was provided by *in vivo* EPR analysis of *E. coli* cells overexpressing the NorR protein in the presence of NO [13]. Cultures induced for NorR expression grown in the presence of NO gave an EPR signal in the $g = 4$ region ($g = 4.19$ and 3.82) suggesting the presence of a non-haem mononuclear Fe–NO complex. These $g$ values are consistent with the formation of a penta- or hexa-co-ordinate $\{Fe(NO)\}^7$ ($s = 3/2$) complex as the sensing mechanism of NorR [13,36]. The $g$ values obtained from these experiments closely matched those of NO-treated isopenicillin N-synthase [37]. In this case, NO was used as a spectroscopic probe to facilitate the analysis of the otherwise EPR-silent non-haem ferrous iron found in the active site of the enzyme. Indeed, NO is often used as a spectroscopic probe for studying a variety of EPR-silent iron-containing proteins such as phenylalanine hydroxylase [38]. The use of *in vivo* EPR provides the advantage that the overexpressed NorR is apparently maintained in the oxygen-sensitive ferrous state by the *E. coli* redox machinery. This permits the rapid screening of site-directed mutants without the requirement for anaerobic purification [13,39]. Indeed, it was possible to rapidly identify the iron-containing domain of NorR and one of the ligands to the iron centre ($Asp^{99}$) using this method. While EPR is still relatively specialized compared with other spectroscopic techniques, the lack of interference caused by sample absorbance makes *in vivo* analysis of Fe–NO complex formation a powerful tool. Combined with *in vivo* complementation assay data, it was possible to prioritize the different NorR GAF domain mutants for further analysis [39].

Subsequent analysis of the ferrous iron centre in the NorR GAF domain required purification of the protein under strict anaerobic conditions. This allowed further biochemical and spectroscopic analysis. EPR was again used with these purified NorR variants to identify any changes in the $Fe^{2+}$-binding pocket of the GAF domain [39]. Identification of candidate ligands to the $Fe^{2+}$ centre in the NorR GAF domain was initially based on the level of conservation and physicochemical properties of individual residues. The absence or alteration of the EPR signal of these nitrosylated variant NorR proteins, combined with *in vivo* data were used to inform structural modelling of the $Fe^{2+}$-containing NorR GAF domain. Taken together, these observations suggest that the $Fe^{2+}$-co-ordinating residues consist of three aspartate residues, a cysteine residue and an arginine residue. Our data fit well with iron content measurements made with site-directed variants of the *Ralstonia eutropha* NorR protein [40]. Interestingly, we also observed significant changes in the EPR spectra with a substitution (Y98L) that is predicted to be located outside of the iron-binding pocket [39]. It is important to note that EPR alone could not rule out a potential role of $Tyr^{98}$ as a ligand to the $Fe^{2+}$ centre. However, the use of UV–visible absorbance spectroscopy provided this evidence, since an LMCT (ligand–metal charge transfer) peak for Tyr-Fe was not detected using this method. UV–visible spectroscopy again proved to be a valuable addition to EPR for the analysis of cobalt-reconstituted NorR to provide further structural insights. In this case, a cobalt–cysteine LMCT peak was observed, confirming the prediction that $Cys^{113}$ is a ligand to the iron centre. This observation was further supported by the use of MCD (magnetic CD) spectroscopy [39].

By combining these observations it has been possible to model the structure of the NorR GAF domain based on the published structure of the mouse PDE2a protein. We favour a hexa-co-ordinate model of the iron centre with five ligands, where one of these ligands is bidentate. The current model of NO-dependent activation of NorR suggests that NO binding to the ferrous iron centre in the NorR GAF domain leads to a ligand-displacement event. This presumably leads to a significant conformational change that allows activation of NorR. As a member of the bacterial enhancer-binding protein family, NorR contains an AAA+ (ATPase associated with various cellular activities) domain that is responsible for stimulating the isomerization of RNA polymerase-containing sigma 54. In the absence of NO, the GAF domain represses AAA+ activity via intramolecular repression. Recent work has focused on identifying the interaction surface between the GAF and AAA+ domains, revealing the region of conformational change and the key residues involved in NO sensing [41,42].

## Spectroscopic analysis of the NsrR NO-sensing mechanism

Spectroscopy has also been instrumental in the analysis of the Fe–S cluster of the NsrR regulatory protein. The NsrR protein from *B. subtilis* has been demonstrated to co-ordinate a 4Fe–4S cluster, while the *Neisseria gonorrhoeae* and *Streptomyces coelicolor* NsrR proteins have been demonstrated to sense NO via a 2Fe–2S cluster [14–16]. This apparent discrepancy could simply represent the evolutionary divergence between NsrR orthologues or may arise from differing purification strategies, although all proteins were produced from *E. coli* [12]. It should also be noted that the anaerobically purified *S. coelicolor* NsrR is capable of co-ordinating a [4Fe–4S] cluster although this form of the protein is unstable when exposed to NO or oxygen [12]. The *S. coelicolor* NsrR protein was initially purified aerobically from *E. coli* and was dark brown in colour, suggesting the presence of an iron cofactor in the protein. UV–visible revealed the broad spectral features of a [2Fe–2S] cluster similar to that found in *E. coli* ferredoxin [14,43]. Subsequent EPR analysis of NO-treated *Streptomyces* NsrR protein revealed the formation of a $s = 1/2$ mononuclear DNIC (dinitrosyl iron complex) ($g = 2.039$, 2.0231 and 2.013) [14]. This observation is consistent with NO splitting the cluster in half, suggesting the sensing mechanism of NsrR as DNIC formation at cysteinyl ligands. Cysteine is the most commonly observed Fe–S cluster ligand and it should be noted that NsrR proteins generally have only three cysteine residues. It has been suggested that the fourth

(non-cysteinyl) ligand could be a conserved histidine residue ($His^{42}$) in the NsrR helix–turn–helix DNA-binding domain. Purified NsrR mutants H42A and C93A have an iron content similar to that of the wild-type protein; however, C99A, C105A and cysteine-free NsrR all exhibited significantly lower iron content when compared with wild-type NsrR (C. Bellota-Antón and N.P. Tucker, unpublished work). One interpretation of these observations is that a single 2Fe–2S cluster is co-ordinated between two NsrR subunits in the functional dimer, requiring only two cysteine residues per monomer. However, MCD analysis revealed spectroscopic similarities to Rieske-type [Fe–S] clusters that have non-cysteinyl ligands such as histidine [14].

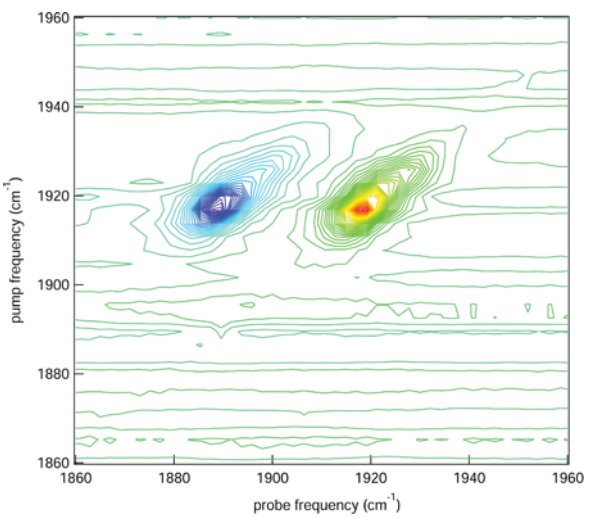
The *B. subtilis* NsrR protein has been subjected to the most comprehensive spectroscopic analysis, including EPR, resonance Raman and UV–visible [15]. Unlike *Streptomyces* NsrR, UV–visible and resonance Raman spectroscopy demonstrated that the *Bacillus* NsrR protein binds a [4Fe–4S] cluster. EPR was also used and demonstrated that a dinitrosyl iron complex was formed on treatment with NO. Interestingly, mixed DNIC complexes were observed at different NO concentrations, implying a graded NsrR response to different levels of nitrosative stress [15]. Recent attention has focused on the DNA-binding activity of site-directed mutants of the *B. subtilis* and *N. gonorrhoeae* NsrR proteins, revealing a significant loss of DNA-binding and repression activity in the cysteine mutants [16,44]. The *B. subtilis* C100A mutant (equivalent to C99A in *S. coelicolor*) has been demonstrated to lack iron and DNA-binding activity [44]. Given that the *S. coelicolor* H42A and C93A mutants both appear to retain iron, spectroscopic analysis will be required to provide insights into the relative contributions of the various Fe–S co-ordinating ligands to the NsrR-sensing mechanism.

## 2D-IR spectroscopic analysis of haem–NO complexes

One experimental tool that has shown significant potential for biomolecule applications is ultrafast 2D-IR spectroscopy [45]. This technique has developed over the last decade or so and has its roots in multidimensional NMR experiments that have revolutionized structural biology. By employing a sequence of ultrashort IR laser pulses, 2D-IR spectroscopy is able to spread the molecular response of the molecule over a second frequency axis in a similar fashion to 2D-NMR experiments. This results in the peaks found in the 1D-IR (one-dimensional IR) spectrum (FTIR) appearing on the 2D-IR diagonal, whereas the off-diagonal region shows peaks that arise from vibrational coupling of individual modes. The technique and experimental procedures for carrying out 2D-IR spectroscopy have been reviewed extensively elsewhere [26,46,47]. The main advantages of 2D-IR in relation to protein studies lies in the high time resolution, typically in the region of 100 fs, which allows access to the hydrogen bond dynamics and ultrafast fluctuations that are not accessible

**Figure 1 | FTIR spectra obtained for equine heart ferric Cc Fe–NO complex**

Results were acquired with a waiting time of 4.5 ps and clearly show two negative peaks (at pump frequency = probe frequency = 1917 and 1927 $cm^{-1}$) on the spectrum diagonal attributable to the $v = 0$–1 transitions of the two structural subsites of the Fe–NO moiety. The positive (blue) peaks shifted to lower probe frequency values correspond to signals from the $v = 1$–2 transitions of the NO-stretching vibration. The spectrum presented here was obtained using a photon echo method that provides enhanced spectral and temporal resolution when compared with previous double-resonance studies [26,53].




by NMR and which are thought to play an important role in controlling structural changes, intermolecular interactions and the solvation state of the protein. In addition, the ability of 2D-IR to determine off-diagonal coupling patterns that arise from different secondary-structural motifs allow a deeper understanding of the inter-residue coupling and vibrational dynamics than is possible through FTIR [47]. This is of interest, because it has been shown that models that accurately simulate FTIR spectral lineshapes for proteins break down when used to mimic the off-diagonal regions of a 2D-IR spectrum, indicating that our knowledge and understanding of these complex molecules is still limited [48]. This combination of structural insight and the ability to resolve protein motion in real time mean that 2D-IR has clear advantages over FTIR spectroscopy.

While still very much in its infancy in terms of biological applications, 2D-IR has shown considerable promise in studies that range from the structural and vibrational dynamics of simple peptides and determination of the timescales for switching between structural substrates in an enzyme, to real-time observation of amyloid fibril formation and temperature-induced protein folding [26,47,49–52]. 2D-IR methods have been used to examine the spectroscopy and dynamics of Fe–NO complexes in haem-containing proteins [53]. By obtaining the 2D-IR spectrum of the N–O stretching mode of the NO complexes of ferric Mb and Cc (for an example, see Figure 1) it was possible to use analysis of

the 2D lineshape to determine the spectral diffusion of the NO ligand caused by electrostatic fluctuations of the haem pocket environment (see Supplementary Movie S1 available at http://www.biochemsoctrans.org/bst/039/bst0391293add.htm). In the 1D-1R spectrum, this is observed as static inhomogeneous line broadening of the NO-stretching vibration but 2D-IR spectroscopy, with its inherent high-time resolution is able to evaluate the dynamics of the equilibrium fluctuations of the protein environment. It was found that the Cc pocket was stiffer than that of Mb, which correlated extremely well with an increased timescale for ligand rebinding of the NO following photolysis from the haem in Mb as compared with Cc. These results indicate the possibility of a direct link between ultrafast protein fluctuations and ligand-binding dynamics. This work demonstrates that NO is a valuable probe for the ultrafast analysis of metalloproteins as well as the prospect of high-time resolution functional studies of NO sensors. Given the large conformational change that apparently results from NO binding to proteins such as NorR [41,54], recent work has focused on spectroscopic methods that provide both structural and temporal insights into NO sensing. The goal of this work is to enable the detection of structural changes in real time in response to NO binding.

## Conclusions and perspectives

It is clear that the diversity of NO-sensing proteins requires a combination of complementary spectroscopic methods to provide insights into the mechanisms of these important proteins. UV–visible, EPR and resonance Raman remain the most commonly used spectroscopic techniques for these studies, but 2D-IR has recently demonstrated great potential. The availability of photochemically activated ruthenium and manganese NO donors provide us with tools for the analysis of NO signalling in real time [55]. The exciting prospect of viewing real-time NO signalling switches in protein structures becomes possible by combining 2D-IR with these NO donors and TEASE (ten-amino-acid-selective and extensive labelling) protein labelling strategies [56].

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