CHAPTER TEN

# Oxygen-Sensing Histidine-Protein Kinases: Assays of Ligand Binding and Turnover of Response-Regulator Substrates

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## Abstract

Heme-based sensors are a recently discovered functional class of heme proteins that serve to detect physiological fluctuations in oxygen ($O_2$), carbon monoxide (CO), or nitric oxide (NO). Many of these modular sensors detect heme ligands by coupling a histidine-protein kinase to a heme-binding domain. They typically bind O2, CO, and NO but respond only to one of these ligands. Usually, they are active in the ferrous unliganded state but are switched off by saturation with O2. The heme-binding domains of these kinases are quite varied. They may feature a PAS fold, as in the *Bradyrhizobium japonicum* and *Sinorhizobium melitoti* FixL proteins, or a GAF fold, as in the *Mycobacterium tuberculosis* DevS and DosT proteins. Alternative folds, such as HNOB (also H-NOX), have also been noted for such signal-transducing kinases, although these classes are less well studied. Histidine-protein kinases function in partnership with cognate response-regulator substrate(s): usually transcription factors that they activate

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by phosphorylation. For example, FixL proteins specifically phosphorylate their FixJ partners, and DevS and DosT proteins phosphorylate DevR in response to hypoxia. We present methods for purifying these sensors and their protein substrates, verifying the quality of the preparations, determining the $K_d$ values for binding of ligand and preparing sensors of known saturation, and measuring the rates of turnover ($k_{cat}$) of the protein substrate by sensors of known heme status.

## 1. Introduction

The FixL-FixJ two-component regulatory system is a paradigm for $O_2$ signal transduction (Gilles-Gonzalez, 2001; Gilles-Gonzalez and Gonzalez, 2005). This simple and classical system consists of only the sensory kinase, FixL, and its substrate and partner, FixJ, each of which is modular (David *et al.*, 1988; Gilles-Gonzalez *et al.*, 1991, 1994; Monson *et al.*, 1992). In FixL, a heme-binding domain couples to a histidine-protein kinase such that the unliganded (deoxy, $Fe^{II}$) form is the "on-state" active kinase, and the oxygen-bound ($K_d$ ~50–140 $\mu M$) form is the "off state" (Gilles-Gonzalez and Gonzalez, 1993; Lois *et al.*, 1993; Mukai *et al.*, 2000). The FixL-catalyzed phosphorylation of a receiver domain in FixJ promotes this response regulator to a dimeric form that activates transcription of target genes (Agron *et al.*, 1993; Da Re *et al.*, 1999; Galinier *et al.*, 1994; Reyrat *et al.*, 1993).

The best-studied FixL–FixJ systems are those that control nitrogen fixation in *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* (formerly *Rhizobium meliloti)* (Dixon and Kahn, 2004; Fischer, 1994; Gilles-Gonzalez and Gonzalez, 2005). Although all ligands of ferrous heme bind to *Bj*FixL and *Rm*FixL, $O_2$ abolishes their kinase activity (>200-fold inhibition), but carbon monoxide (CO) and nitric oxide (NO) do not (less than threefold inhibition) (Dunham *et al.*, 2003; Gilles-Gonzalez *et al.*, 1994; Sousa *et al.*, 2007a). The unliganded form of ferric *Bj*FixL is fully active, and cyanide inhibits this species analogously to the $O_2$ inhibition of the ferrous form (Gilles-Gonzalez *et al.*, 2006). The electronic resemblance between the cyanomet species ($Fe^{III}CN^-$) and the substantively polarized $O_2$-bound state ($Fe^{II}O_2 \cdot Fe^{III}O_2^-$), together with crystal structures of liganded forms of the heme-binding domain, suggest that regulatory switching of the kinase is triggered by changed polar interactions of the heme with residues of the heme pocket (Gong *et al.*, 1998; Hao *et al.*, 2002; Olson and Phillips, 1997).

As a rule, microbial $O_2$ sensors serve to initiate substantive lifestyle changes. For example, hypoxia is a key determinant of the rhizobial switch from a vegetative to a nonreplicative symbiotic state (David *et al.*, 1988; Ditta *et al.*, 1987; Sciotti *et al.*, 2003; Soupene *et al.*, 1995; Virts *et al.*, 1988). In low $O_2$, rhizobial FixL–FixJ systems induce a cascade of gene (*nif, fix*) expression that produces the nitrogen-fixation enzymes and their accessory proteins,

the key regulators of denitrification, and one or more high-affinity alternative terminal oxidases for respiration in low $O_2$. The latter function, which enables respiration during hypoxia, is also reported for homologs of FixL and FixJ in microorganisms that do not fix nitrogen (Crosson *et al.*, 2005). In addition to FixLs, several classes of histidine-protein kinases employ alternative heme-binding domains to sense gaseous ligands (Gilles-Gonzalez and Gonzalez, 2005). For example, the *Mycobacterium tuberculosis* DevS and DosT hypoxia sensors couple their kinase to a heme-binding GAF domain instead of the heme-binding PAS domain found in FixLs (Ioanoviciu *et al.*, 2007; Sardiwal *et al.*, 2005; Sousa *et al.*, 2007b). Like FixL, these *M. tuberculosis* sensors phosphorylate a response-regulating transcription factor (DevR), and the resulting broad changes in gene expression trigger a state of nonreplicative persistence: in this case a latent infection of a human host (Roberts *et al.*, 2004; Saini *et al.*, 2004a,b). Clearly, the readily accessible ligand-binding and enzymatic parameters of heme-based $O_2$ sensors make them ideal subjects for studies of signal transduction, and their involvement in relevant lifestyle changes of bacteria lends additional significance to their study.

## 2. Assays

### 2.1. General considerations

#### 2.1.1. Autophosphorylation

If a histidine-protein kinase is supplied with its ATP but not its protein substrate, it slowly (10–40 min) converts itself to a phosphorylated species (Hess *et al.*, 1991; Stock *et al.*, 1989). For example, the addition of ATP to deoxy-FixL yields a phospho-FixL species (Gilles-Gonzalez and Gonzalez, 1993; Tuckerman *et al.*, 2001).

$$\text{Autophosphorylation} \quad \text{FixL2} + 2\ \text{ATP} \leftrightarrows \text{P-FixL}_2 + 2\ \text{ADP}$$

The so-called autophosphorylation is really a trans-phosphorylation between the subunits of these requisitely dimeric kinases (Ninfa *et al.*, 1993). For sensors such as FixL, this reaction will reliably report *qualitative* information on

- Ligands that regulate the kinase
- Preferred divalent cation for the enzyme
- Contamination of the enzyme preparation with a phosphatase

However, this reaction cannot give a quantitative measure of the enzymatic activity or its regulation because it is not a valid, rate-limiting, half-reaction. Contrary to the ping-pong bi–bi mechanism often presumed for many histidine-protein kinases, FixL does not process its nucleotide and protein substrates independently (Tuckerman *et al.*, 2001, 2002). FixJ clearly enhances the initial FixL phosphorylation. For example, phosphorylation of *Rm*FixL is accelerated about eightfold by the inclusion of an unphosphorylatable FixJ (the D54N *Rm*FixJ variant) in the reaction (Sousa *et al.*, 2005). More importantly, even in a large excess of ATP the reaction of FixL with only ATP stops when only about 20% of FixL is phosphorylated. In contrast, formation of the phosphorylated FixL intermediate proceeds essentially to completion if the true intermediate is trapped by including the unphosphorylatable FixJ substrate in the reaction (Sousa *et al.*, 2005). Finally, when FixJ is added to phospho-FixL ''preformed'' by autophosphorylation, very little phosphoryl transfer occurs; instead, most of the phosphorylated protein is hydrolyzed to free phosphate. These observations imply that the ''phospho-FixL'' produced by these two methods are not kinetically equivalent (Tuckerman *et al.*, 2001). This may also be the case for many other sensory kinases presumed to carry out their phosphoryl transfers sequentially and reported to show highly inefficient phosphoryl transfer to their protein substrate under those conditions.

### 2.1.2. Turnover

Since FixL-catalyzed phosphoryl transfers clearly take place with FixL, FixJ, and ATP present, the turnover rate $k_{cat}$ provides the best measure of the FixL reaction kinetics and the effects of regulatory ligands on those kinetics (Sousa *et al.*, 2007a; Tuckerman *et al.*, 2002).

$$\text{Turnover:}\quad 2\ \text{FixJ} + 2\ \text{ATP} \xleftrightarrows{FixL_2} \text{P-FixJ}_2 + 2\ \text{ADP}$$

This rate represents the number of molecules of a specific substrate that one molecule of a specified form of an enzyme will phosphorylate per minute while it is saturated with all of its substrates. Consequently, an accurate $k_{cat}$ measurement requires that the kinase be kept at levels sufficiently high to ensure preservation of its dimeric state and yet sufficiently low to guarantee that the level of enzyme–substrate complex will not change during the measurement, i.e., the time for the enzyme to reach steady state and complete at least 10 turnovers. For the FixL–FixJ system, this means about 1 $\mu M$ FixL, $>20$ $\mu M$ FixJ, and 1.0 m$M$ ATP. For $k_{cat}$ determinations, it is also essential to collect complete time courses so that the portion the time course may be found where product accumulates at a

constant rate (i.e., when the rate of phosphorylation of the protein substrate matches the rate of replenishment of the phosphorylated enzyme intermediate). For inhibited states of the enzyme (e.g., FixL partially saturated with $O_2$), there can be a lag while the enzyme intermediates build up. For highly active states (e.g., deoxy-FixL), there can be an early deceleration of the reaction rate due to rapid depletion of substrate. An informative indicator of the efficacy of a heme ligand is its *inhibition factor*, defined as the ratio of turnover rates of the unliganded and the fully liganded forms for the same oxidation state. For low-affinity native and mutant sensors, saturation with $O_2$ may not be practical. In these cases, great caution should be used in estimating the activity of a hypothetical fully oxygenated sensor, as the activity of partially saturated mixtures is not necessarily the sum of the activities of the liganded and unliganded species weighted by their relative abundance (Sousa *et al.*, 2007a). In addition to the aforementioned considerations about steady state, determinations of inhibition factor require verifying that the heme status (saturation, oxidation state) remains unchanged throughout the measurements of reaction kinetics.

## 2.2. Protein purifications

### 2.2.1. Strategy for purifying heme-containing histidine-protein kinases

Sensory kinases such as FixL and DosT are easily monitored from their intense red color. They readily yield to traditional methods of protein fractionation, with an anion-exchange step giving the most significant purification because of their unusually low isoelectric points (p*I* 5–6) (Scopes, 1994). Moderate overexpression of the corresponding genes at about 5% of total cell proteins from an inducible *Escherichia coli* promoter (e.g., *tac*) usually gives the highest yield of soluble heme protein. While it is possible to obtain much higher expression with some vectors, the resulting protein is usually of low quality, with weak enzymatic activity and a tendency to aggregate. It is far easier to grow more cells than to try to recover activity from misfolded or aggregated protein. Typically, a 4-liter culture of an *E. coli lacI*$^q$ strain (e.g., TG1) harboring the gene on a plasmid is grown overnight in a fermentor (Bioflow 3000) at 37°, 200–500 rpm, and 20% of atmospheric $O_2$. When the culture reaches an $OD_{600}$ of about 0.5, expression of the heme protein is induced with isopropyl-$\beta$-D-thiogalactoside (1 m*M*). When growth of the cells begins to slow (cell density about 30 g/liter), they are cooled to 4°, harvested, and lysed by sonication in 2 volumes of lysis buffer [20 m*M* Tris-HCl, pH 8.0, 100 m*M* NaCl, 3 m*M* KCl, 1 m*M* EDTA, 10 m*M* β-mercaptoethanol, 0.04 mg/ml lysozyme, 0.17 mg/ml phenylmethylsulfonyl fluoride (added at room temperature from a 40-mg/ml solution in acetone)]. The lysate is cleared by centrifugation at 70,000 rpm (Ti 70 rotor, 30 min, 4°).

The clear-red lysate, kept at about 4°, is slowly brought to 1.2 *M* ammonium sulfate with stirring and dropwise addition of 4.0 *M* ammonium sulfate, 2 m*M* EDTA, pH 7.5, and centrifuged at 12,000 rpm (SS43 rotor, 4°). Assuming the pellet density to be about 1 g/ml, the red precipitate is diluted threefold with 50 m*M* Tris-HCl, 50 m*M* NaCl, 5% (v/v) glycerol, and 10 m*M* $\beta$-mercaptoethanol, pH 7.5, and desalted on a size-exclusion column (Sephadex G-25, GE Healthcare) preequilibrated with 50 m*M* Tris-HCl, 50 m*M* NaCl, 5% (v/v) glycerol, and 10 m*M* $\beta$-mercaptoethanol, pH 7.5, at 4°. Subsequent tracking of the heme protein is done automatically from its 415-nm absorption (Bio-Rad QuadTec UV/vis detector). The protein mixture is chromatographed on an anion-exchange column (DEAE-Sephacel, Amersham), with thorough washing in 100 m*M* NaCl, and elution from a gradient of 100–300 m*M* NaCl in 50 m*M* Tris-HCl, 5% (v/v) glycerol, and 10 m*M* $\beta$-mercaptoethanol, pH 7.5, at 4°. The heme protein-containing fractions are further purified (to about 95% purity) by gel filtration (Superdex S-200, GE Healthcare) on a column preequilibrated with 50 m*M* Tris-HCl, 50 m*M* NaCl, and 5% (v/v) glycerol, pH 8.0, at 4°. Depending on the stability to oxidation of the purified protein, it will be a mixture of $Fe^{III}$ and $Fe^{II}O_2$ states, or entirely in the $Fe^{III}$ state (Gonzalez *et al.*, 1998). Concentrate the protein to about 100 $\mu M$ in a filtration unit (Amicon, 10-kDa membrane cutoff) and store in aliquots of about 0.5 ml each at −70°.

### 2.2.2. Quality control

***Heme content*** The heme content of the purified proteins may be quantified by a pyridine hemochromogen assay, with hemin as the standard (Appleby, 1980). When bacterial heme-containing histidine-protein kinases are overproduced in *E. coli*, they are typically recovered with their full complement of heme (one heme per monomer). For comparison to the heme content, the protein concentration may be measured by the BCA protein assay (Pierce Biotechnology Inc.) with bovine serum albumin as the standard.

***Preparation of deoxy protein*** Incubate the purified protein (~100 $\mu M$) for about 15 min with an anaerobic solution of 10 m*M* dithiothreitol in 50 m*M* Tris-HCl, 50 m*M* KCl, 5.0% (v/v) ethylene glycol, and 1 m*M* $MgCl_2$, pH 8.0, inside of an anaerobic chamber (Coy Laboratory Products, Inc.). This procedure works well for heme-based sensors of relatively low redox potential (e.g., FixL, EcDos, DosT) and converts them fully to the deoxy state. If this approach does not yield the deoxy state within 15 min, reduce the protein with 1 equivalent of the stronger reducing agent sodium dithionite inside the anaerobic chamber and immediately remove this chemical and its by-products by gel filtration on a Sephadex-G25 column (about 3 ml) equilibrated with the same buffer as described earlier. If the protein is to be used in the deoxy state or mixed with CO, keep it in the aforementioned buffer. If the protein is to be mixed with $O_2$, dilute

the solution to less than 1 m*M* dithiothreitol; if it is to be mixed with NO, remove all of the reducing agent. Verify the quality of the preparation by recording the 350- to 700-nm absorption spectrum.

*Exploiting autophosphorylation to verify phosphatase contamination* Transfer the deoxy-protein solution to a sealable cuvette and measure its 350- to 700-nm absorption. Assuming the 434-nm extinction ($\varepsilon_{434}$) of the deoxy state to be about 130 $mM^{-1}cm^{-1}$, adjust the protein concentration to 4–5 $\mu M$ in 50 m*M* Tris-HCl, 50 m*M* KCl, 5.0% (v/v) ethylene glycol, and 1 m*M* $MgCl_2$, pH 8.0, and equilibrate it at 23°. Separately prepare a series of Eppendorf tubes labeled for each time point and each containing 3.3 $\mu$l of "stop buffer" [10 m*M* EDTA, 2% (w/v) sodium dodecyl sulfate, 0.40 *M* Tris-HCl, 50% (v/v) glycerol, and 2% (v/v) $\beta$-mercaptoethanol, pH 6.8]. Begin the reactions by introducing ATP to a final concentration of 1.0 m*M* (unlabeled ATP from Sigma and [$\gamma$-$^{32}$P]ATP from Amersham Pharmacia Biotech, specific activity 0.21 Ci/mmol). At timed intervals (e.g., 1.5, 3.0, 6.0, 12, 24, and 48 min), withdraw a reaction aliquot from the cuvette (10 $\mu$l) and mix it rapidly with the stop buffer (3.3 $\mu$l) from the appropriate tube.

The stopped reactions may now be transferred to air. Electrophorese 10 $\mu$l from each time point on an 11% (w/v) SDS polyacrylamide gel (Fig. 10.1A) (Laemmli, 1970). Spot 1 $\mu$l from the later time points (e.g., 12, 24, 48 min) onto a polyethyleneimine-cellulose thin-layer chromatographic (TLC) plate, about 2 cm from the bottom and 1 cm from the next time point; include a control on the plate containing only the radiolabeled ATP (Fig. 10.1B). After air drying the plate, develop it in a sealed TLC container with a 1-cm layer of 0.75 *M* $NaH_2PO_4$, pH 3.5. Quantify the levels of phosphorylated protein in the dried gels and of low-molecular weight species on the TLC plates with a phosphorimager (Bio-Rad Personal Molecular Imager FX). As standards for the quantification, spot 1-$\mu$l 10-fold dilutions of the stock ATP solution onto a strip of cellulose for development alongside the gel and TLC. If the protein is free of phosphatase, the TLC will show no significant generation of free phosphate (Pi) in 45 min, and the autoradiograph will show a continuous increase or a leveling of the autophosphorylation over the same period (Fig. 10.1). If the protein is contaminated with a phosphatase, free phosphate will be obvious from the TLC, and the level of protein phosphorylation will decline after reaching an apparent peak. For such preparations, further purification is advised.

*Verification of kinase activity* The absence of contaminating phosphatase activity is necessary but not sufficient to ensure that the entire enzyme in a preparation is active. Neither is the initial rate of autophosphorylation a reliable indicator of enzyme quality. A healthy kinase preparation should yield about 20% phosphorylated kinase at equilibrium. If a modified protein

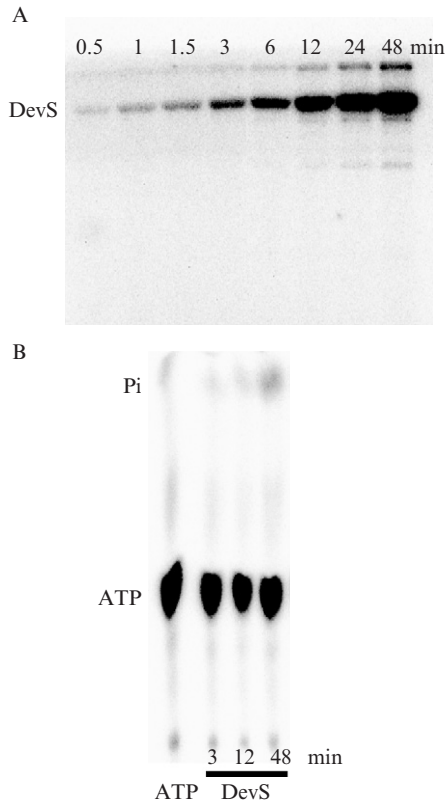


**Figure 10.1** Quality of the *M. tuberculosis* $O_2$ sensor DevS is verified readily by autophosphorylation. The increasing autophosphorylation of deoxy-DevS even after 24 min from the autoradiograph (A) and the insignificant production of free phosphate even in 48 min from the polyethyleneimine TLC plates (B) show this preparation to be free of phosphatase contamination. From Sousa *et al.* (2007b).

substrate unable to accept phosphate is used, phosphorylation of the kinase should proceed nearly to completion. Any enzyme that does not meet these standards should be discarded if one wishes to have measurements that are both accurate and reproducible from batch to batch.

### 2.2.3. Strategy for purifying response-regulator substrates

These proteins are best isolated by affinity purification. We have found that an epitope-tagged 6-His (Invitrogen) introduced at the N-terminal end of the gene by recombinant DNA methods works well for purifying these response regulators. The protein is purified as recommended by the manufacturer of the tag and affinity column (nickel-charged affinity resin) and assayed based on Western blotting and antibody recognition of the tag

(Invitrogen). For quality control, examine phosphorylation of the protein in an extended turnover assay, with time points ranging from 1 to 30 min (see later). Quantitative phosphorylation of response regulators (i,e., FixJ) entails a phosphorylation in one subunit of a dimer (Da Re *et al.*, 1999). Acceptable preparations of kinase and protein substrate should result in about 50% phosphorylation of the protein substrate at equilibrium (Sousa *et al.*, 2007a).

## 2.3. Measuring the $K_d$ for binding of ligand

### 2.3.1. Direct titration with O2

***Preparation of O2-saturated buffer*** Prepare a solution of 1.3 m*M* $O_2$ in 50 m*M* Tris-HCl, 50 m*M* KCl, and 5% (v/v) ethylene glycol at pH 8.0 by bubbling pure $O_2$ for about 30 min through this buffer at room temperature (23°) in a septum-sealed glass vial with a needle and an escape. Move the sealed vial to an anaerobic chamber. Transfer aliquots of the $O_2$ solution from the vial to anaerobic buffer or the deoxy protein, as necessary, with a gas-tight Hamilton syringe.

***Basis spectra and exploratory titration*** Before starting a ligand titration, it is essential to record absorption spectra of the protein at 0 and 100% of saturation with the ligand. These spectra will serve as basis spectra for data analysis and will make clear the boundaries for titration. Prepare deoxy protein (4 $\mu M$) as described earlier; place the protein solution in a septum-sealed cuvette and record its 350- to 700-nm absorption spectrum. Add a concentrated aliquot of the deoxy heme protein (final concentration 4 $\mu M$) to a septum-sealed solution of $O_2$-saturated buffer (1.3 m*M* $O_2$) with a gas-tight syringe and record the 350- to 700-nm absorption. Record both spectra in an Excel spreadsheet. An optimum linear combination of these spectra may be used to fit the spectrum resulting from each experimental titration (acceptable overall fit should be $>0.98$) and generate a percentage of saturation. If the spectrum cannot be closely fit to a linear combination of liganded and unliganded spectra, this means that some process other than ligand association is occurring and that some other species is contributing to spectra collected during titration. Most commonly, this means that the heme iron is oxidizing during titration, and the error spectrum is that of the ferric state. The reason that whole spectra should always be fitted, rather than a few wavelengths, is to detect the generation of extra species during the titration. One should not attempt to correct for oxidized protein or any third species; instead, find a way to control the interfering process and repeat the titration.

Heme-based sensor variants with $K_d$ values for $O_2$ ranging from millimolar to submicromolar may be encountered; usually, there is no previous information on the expected affinity for $O_2$. Therefore, a quick exploratory titration is recommended. Add five $O_2$ concentrations, ranging

from about 1 to 600 $\mu M$ $O_2$, sequentially to the protein within the same cuvette, minimizing the headspace as much as possible, and record the absorption spectrum resulting from each $O_2$ concentration. Estimate the point of half saturation and design an experiment for exploring the most informative range of $O_2$ concentrations ($O_2$ required to give 10–100% saturation).

***Full titration*** A full titration experiment might require about 10 $O_2$ concentrations to cover the range for 10–100% saturation and should be repeated at least twice (Fig. 10.2). To minimize escape of gases or concurrent reactions (e.g., oxidation), $O_2$ titration points should be prepared individually and their absorption recorded immediately. For example, prepare a solution of a known $O_2$ concentration (e.g., 2.0 $\mu M$ $O_2$); combine it with deoxy protein (1–4 $\mu M$ final) in a 1-cm path length cuvette, taking care to minimize the headspace, and immediately seal the cuvette. Record

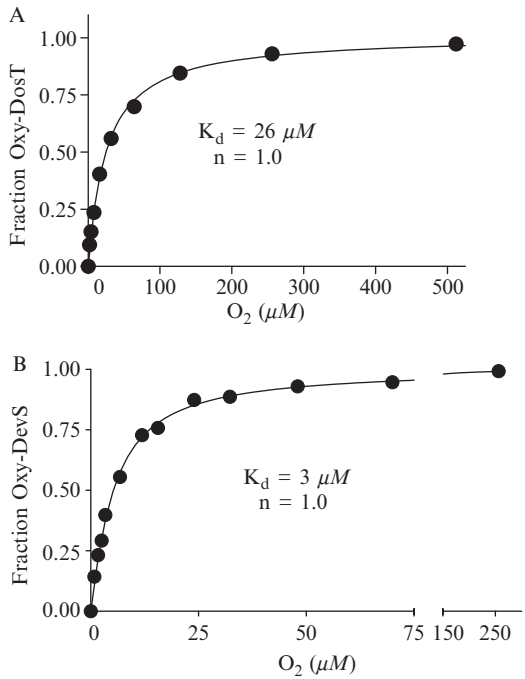


**Figure 10.2** The $K_d$ for binding of $O_2$ can be directly determined by titration with ligand. (A) Ferrous *M. tuberculosis* DosT (2 $\mu M$) was titrated with 1–1200 $\mu M$ $O_2$ at pH 8.0 at 25°. Saturation data were fit to a nonlinear Hill equation from which the $K_d$ and Hill coefficient $n$ were determined. (B) The $K_d$ for binding of $O_2$ to the higher-affinity *M. tuberculosis* DevS protein was determined similarly by titrating the deoxy form (2 $\mu M$) with 0.8–256 $\mu M$ $O_2$ under the same conditions as in A. From Sousa *et al.* (2007b).

the 350- to 700-nm absorption of this mixture. Repeat this procedure for all the $O_2$ concentrations covered by titration. If the protein is prone to oxidation, the most challenging aspect of the titration is to make certain that the spectrum for each concentration is recorded after thorough mixing but before any oxidation. Reducing agents to control oxidation should be avoided, especially at low $O_2$ concentrations, as these will consume $O_2$. If it is necessary to use reducing agents, one must verify that the $O_2$ saturation does not change for that specific $O_2$ concentration during the time needed to collect the spectrum. Saturation can be determined by comparing actual spectra to linear combinations of basis deoxy and oxy spectra. Treated data are plotted and fitted to a nonlinear Hill plot equation and to a quadratic single-binding equation using one's favorite curve-fitting software (e.g., Microsoft Excel or GraphPad Prism).

An alternative to titration in a sealed cuvette is to use a system equilibrated with a large headspace of gas (e.g., a tonometer) at a controlled gas concentration. This is how most of the early titrations of hemoglobin and myoglobin were done. This approach requires designing and fabricating custom glassware, but it may be easier than the sealed approach if one has fairly large volumes of protein available.

### 2.3.2. Direct titration with CO

Determination of the $K_d$ value for binding CO can be done by a procedure similar to the aforementioned determination of $O_2$ affinity for most sensors because their $K_d$ values for binding of CO typically fall in the 0.5 to 10 $\mu M$ range. The CO-saturated buffer (1.0 m*M*) is prepared *in a fume hood* by bubbling pure CO for about 30 min through an anaerobic solution of 50 m*M* Tris-HCl, 50 m*M* KCl, and 5% (v/v) ethylene glycol at pH 8.0 and 25° in a gas-washing bottle or any septum-sealed glass vial with a needle and an escape. For basis spectra, record the 350- to 700-nm absorption of the deoxy heme protein (1 $\mu M$) and the identical concentration of ferrous protein in a 1.0 m*M* solution of CO. For titrations, transfer aliquots of the CO solution from a septum-sealed vial to a sealable cuvette with the deoxy protein via a gastight Hamilton syringe.

### 2.3.3. Competition titration with NO

The much higher NO affinity of heme proteins forbids their direct titration with NO. Instead, carbon monoxy protein is prepared at a known CO concentration and then titrated with NO, which displaces the CO (Fig. 10.3). The $K_d$ value for binding of NO to the heme protein can be calculated from the "apparent" $K_d$ in the presence of CO and the precisely known $K_d$ value for binding of CO. Data analysis is the same as that of an ordinary titration, except that the basis spectrum for the unliganded state is replaced by the spectrum of carbon monoxy protein. To prepare the solution of NO-saturated heme protein, first prepare anaerobic buffer [50 m*M*

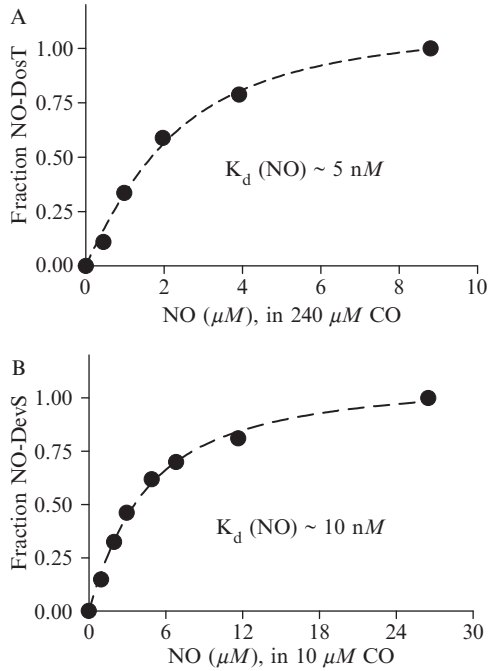


**Figure 10.3** The $K_d$ for binding of NO can be determined by competition against CO. (A) Ferrous *M. tuberculosis* DosT in 240 $\mu M$ CO ($K_{d(CO)} = 0.94$ $\mu M$, $n = 1.0$) was titrated competitively with 0.50–9.0 $\mu M$ NO at pH 8.0 at 25°. (B) Ferrous *M. tuberculosis* DevS in 10 $\mu M$ CO ($K_{d(CO)} = 0.036$ $\mu M$, $n = 1.0$) was titrated competitively with 0.50–28 $\mu M$ NO under the same conditions as in A. From Sousa *et al.* (2007b).

Tris-HCl, 50 m*M* KCl, and 5% (v/v) ethylene glycol at pH 8.0 and 23°] inside of an anaerobic chamber in a vessel fitted with a three-way valve. Maximize the surface area of the solution and do not fill more than one quarter of the vessel with liquid. Seal the vessel, transfer it to *a fume hood*, and connect it to nitrogen and NO tanks via the three-way valve. Briefly flow nitrogen through the line and reservoir and then switch to the NO tank and flow a gentle stream of NO through the reservoir for 30 min. Transfer the NO-saturated buffer (2.0 m*M* NO, 1 ml) from the reservoir to the anaerobic chamber in a gas-tight Hamilton syringe. Prepare a solution of 1 $\mu M$ ferrous protein in 20–40 $\mu M$ NO; the heme protein should be fully saturated with NO.

The very popular NO-generating reagents are not suitable for quantitative analytical work because they do not allow concentrations of NO to be precisely controlled and determined and they generate reactive species other than NO. The importance of handling NO and its solutions properly cannot be overemphasized. The pure gas is extremely reactive and rapidly destroys most metals and plastics. Nitric oxide vessels and tanks should be kept *in a fume*

*hood*. Only polytetrafluoroethylene, silicone rubber, stainless steel, and glass should be used in apparatus that contacts NO. All traces of $O_2$ and other substances that will react with NO must be scrupulously excluded from all solutions. Otherwise, the very remarkable phenomena that you will certainly observe will be only indirectly because of NO. A serious leak will result in nitric acid generation, which can be easily verified by checking the sample pH. One may also generate highly oxidizing species such as peroxynitrite. A sure sign that there is a leak somewhere in the NO-handling apparatus, or that something in the buffer reacts with NO, is that the heme protein absorption spectrum irreversibly changes or the protein denatures. One should also be aware that the pure NO in the supply tank, even in the absence of contaminants, may disproportionate into $N_2O_5$ and $N_2$ over time. In the event that a novel heme protein displays highly unusual behavior toward NO, one should always test a protein control such as myoglobin, whose reactions with NO are thoroughly known, before announcing one's discovery in a press release.

## 2.4. Determination of turnover rates, $k_{cat}$

### 2.4.1. General procedure

For heme-controlled histidine-protein kinases such as FixL, assays of $k_{cat}$ measure the rates at which a sensor of known heme status phosphorylates its protein substrate (Fig. 10.4). Such studies are essential for quantifying the

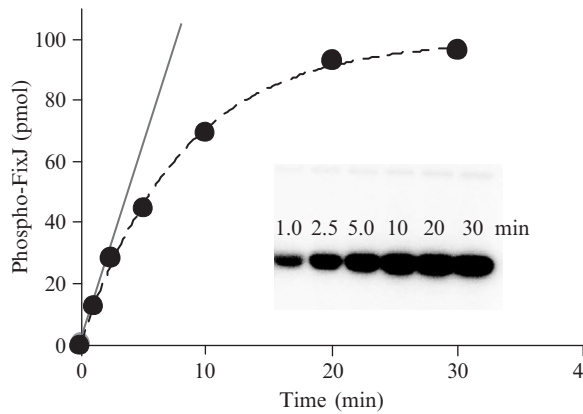


**Figure 10.4** The best measure of kinase activity for *B. japonicum* FixL is its rate of turnover ($k_{cat}$) of FixJ to phospho-FixJ. This reaction was catalyzed by deoxy-*Bj*FixL (1 $\mu M$) for 25 $\mu M$ *Bj*FixJ and 1 m$M$ ATP at pH 8.0 at 23°. The $k_{cat}$ is 1.5 $min^{-1}$ (gray line). Quantitative phosphorylation of FixJ occurred at equilibrium, verifying the excellent quality of the enzyme and substrates, with one-half of the *Bj*FixJ (200 pmol) being phosphorylated within 30 min. From Sousa *et al.* (2007a).

influence of the heme on the kinase. For example, a typical experiment might compare phosphorylation of a response regulator (e.g., FixJ or DevR) by the deoxy, oxy, or carbon monoxy states of its kinase partner (e.g., FixL or DevS). For the turnover of FixJ to phospho-FixJ, reaction mixtures typically contain 1 $\mu M$ FixL and 25 $\mu M$ FixJ in 50 m$M$ Tris-HCl, 50 m$M$ KCl, 50 $\mu M$ $MnCl_2$, 1 m$M$ $MgCl_2$, and 5% (v/v) ethylene glycol, pH 8.0. Reactions are started by introducing the ATP (i.e., unlabeled ATP from Sigma and [$\gamma$-$^{32}$P]ATP of specific activity 0.42 Ci/mmol from Amersham Pharmacia Biotech) (see Fig. 10.4). They are stopped at 1.0, 2.5, 5.0, 10, 20, and 30 min by mixing 10-$\mu$l aliquots of the reaction mixtures with 3.3 $\mu$l of "stop buffer" [10 m$M$ EDTA, 4% (w/v) sodium dodecyl sulfate, 0.50 $M$ Tris-HCl, 0.20 $M$ NaCl, 50% (v/v) glycerol, and 2% (v/v) $\beta$-mercaptoethanol, pH 6.8]. The products are electrophoresed on 15% (w/v) polyacrylamide gels (Laemmli, 1970). The levels of phosphorylated FixJ protein in the dried gels are quantified with a phosphorimager (Bio-Rad Personal Molecular Imager FX). A good preparation of kinase, in the on state, should quantitatively phosphorylate its protein substrate at equilibrium (see Fig. 10.4).

Interpretation of kinetic data such as these critically relies on the assumption that the heme state remains the same throughout every reaction time course. It is therefore essential to verify 350- to 700-nm absorption spectra before and after every time course. It is also important to sample a sufficient number of time points so that each turnover rate may be computed at steady state, i.e., when the rate of FixJ phosphorylation matches the rate of replenishment of the phospho-FixL intermediate. One must additionally ensure that the concentration of all substrates is constant or else well above $K_m$ throughout the period of the reaction time course on which rate measurements are based, as extraction of fundamental rate constants from the reaction time course will otherwise be prohibitively complex and error prone.

### 2.4.2. Sensors with partially saturated heme

Prepare the liganded protein as described earlier. Calculate saturation based on linear regression using whole spectra. Again, verify that the heme state remains unchanged throughout every time course, make sure that the enzyme is saturated with its substrates during the entire reaction, and collect enough data points to ensure coverage of the turnover rates at steady state.

## 2.5. Novel heme-containing histidine-protein kinases

The vast majority of proteins predicted to be heme-containing histidine-protein kinases, or even identified as FixLs from their genetic context or possession of heme, have not been examined for their enzymatic activity and regulation of this activity by heme ligands (Freitas *et al.*, 2003; Gilles-Gonzalez and Gonzalez, 2005; Iyer *et al.*, 2003; Sardiwal *et al.*, 2005;

Taylor and Zhulin, 1999). We anticipate that many of these sensors will soon be studied for signal transduction as the complete enzymes. For initial study of a novel heme-containing kinase, additional considerations should be divalent-cation and heme-state requirements for activity. Although most histidine-protein kinases work well with $Mg^{II}$, others display strong preferences for specific divalent cations (Hess *et al.*, 1991). For example, *S. meliloti* FixL performs best in assay mixtures that include $Mn^{II}$ (50 $\mu M$) (Gilles-Gonzalez and Gonzalez, 1993). Likewise, although all heme-containing kinases examined so far work best in the deoxy state, it is quite possible that this represents the ''off'' state of some sensors, and inclusion of a ligand in the assays is necessary to manifest the ''on'' state.

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