Kinase Activity of Oxygen Sensor FixL Depends on the Spin State of Its Heme Iron†

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ABSTRACT: FixL is a ferrous heme protein whose kinase activity is inhibited by oxygen. Here we show that met-FixL, which is the ferric unliganded form, has the same activity as deoxy-FixL, the ferrous unliganded form, indicating that activity does not depend on the oxidation state of the heme iron. The ferric derivative fluoro-FixL is fully active, indicating that the presence of a heme ligand is not sufficient to cause kinase inhibition. An inverse relation between the rate of autophosphorylation of ferric FixL and the fractional saturation with cyanide shows that the cyanomet form has zero activity. All our active derivatives were high-spin, while our inactive derivatives were low-spin. In mixtures of high- and low-spin FixL, resulting from partial saturation with low-spin ligands, the activity was that which would be expected for the concentration of the high-spin component alone. Therefore the spin state of the heme iron rather than the oxidation state or presence of ligands must be the factor that controls FixL's kinase activity. On transition from low to high spin, the heme iron moves out of the porphyrin plane by 0.4 Å. We propose that, as in hemoglobin, this motion triggers a long-range conformational change which in FixL is responsible for a switch to an active form.

Oxygen regulates diverse processes essential to life, including red blood cell production, blood flow to the brain, gluconeogenesis, nitrogen fixation, and the transition from glycolysis to oxidative phosphorylation (Goldberg et al., 1988; Ganfornina & Lopez-Barneo, 1992; Kietzmann et al., 1993; Ditta et al., 1987; Guest, 1992; Poyton & Burke, 1992). The FixL proteins that regulate nitrogen fixation in rhizobia are the only oxygen sensors isolated so far (Gilles-González et al., 1991, 1994). They are homologous to a large family of sensor kinases belonging to the two-component regulatory systems [reviewed in Stock et al. (1989) and Parkinson and Kofoid (1992)]. The FixL from *Rhizobium meliloti* is a chimeric heme protein kinase whose enzymatic activity is reversibly blocked by oxygen binding to the heme; oxygen exerts its effect at an initial autophosphorylation step (Gilles-González & González, 1993). FixLs bind oxygen non-cooperatively with very low affinities which enable them to give a linear response of kinase activity over a wide range of ligand concentrations; it seems likely that other heme-based sensors for a variety of heme ligands would have similar properties.

Unlike the *R. meliloti* FixL, which we have been able to study only as a soluble truncation, the heme-containing FixL protein from *Bradyrhizobium japonicum* is soluble in its native form. Here we show that *B. japonicum* FixL is also a kinase. We find that the inhibition of its kinase activity by oxygen is effected by the conversion of the heme iron from high to low spin. High-spin FixL, whether ferrous or ferric, liganded or unliganded, has full kinase activity, while low-spin derivatives are inactive. In hemoglobin, such a spin-state change is accompanied by movement of the heme iron in and out of the porphyrin plane (Perutz, 1970, 1989). This motion is transmitted to the protein through the tightly bound proximal histidine and triggers a change in tertiary structure. A similar spin-driven movement of the proximal histidine in FixL appears to control its kinase activity. This is the first evidence that the sensor (input) domains of sensor kinases belonging to two-component systems control their kinase (transmitter) domains by a long-range conformational change and also the first demonstration of such control by the spin state of a heme domain.

EXPERIMENTAL PROCEDURES

Proteins. B. japonicum FixL was expressed at high levels in *Escherichia coli* strain TG1(pBL31) (Gilles-González et al., 1994). Purification of the protein was essentially as described for the *R. meliloti* FixL* (Gilles-González et al., 1991). The final preparations contained met-FixL in 5 mM Tris-HCl, pH 8.0.

FixL Heme Derivatives and UV−Visible Spectroscopy. All derivatives were in phosphorylation buffer (50 mM NaHepes, pH 8.0, 50 mM KCl, and 0.20 mM $MnCl_2$) at a final concentration of 5−25 μM FixL. Heme ligands were equilibrated with the protein for at least 10 min.

Unless otherwise indicated, cyano-FixL and fluoro-FixL were made by equilibrating met-FixL with 10 mM KCN and 100 mM NaF, respectively. Measurement of cyanide binding was calculated from the absorbances at 424 nm and at the 475-nm isosbestic point.

Deoxy-FixL was prepared by passing a stream of N_2 over a met-FixL solution and then adding a few crystals of sodium dithionite. To produce carbonmonoxy-FixL, the deoxy-FixL was diluted two-fold with CO-saturated phosphorylation buffer. Nitric oxide−FixL was obtained from a mixture of nine volumes of deoxy-FixL with 1 vol of NO-saturated phosphorylation buffer. Alternatively, nitric oxide−FixL

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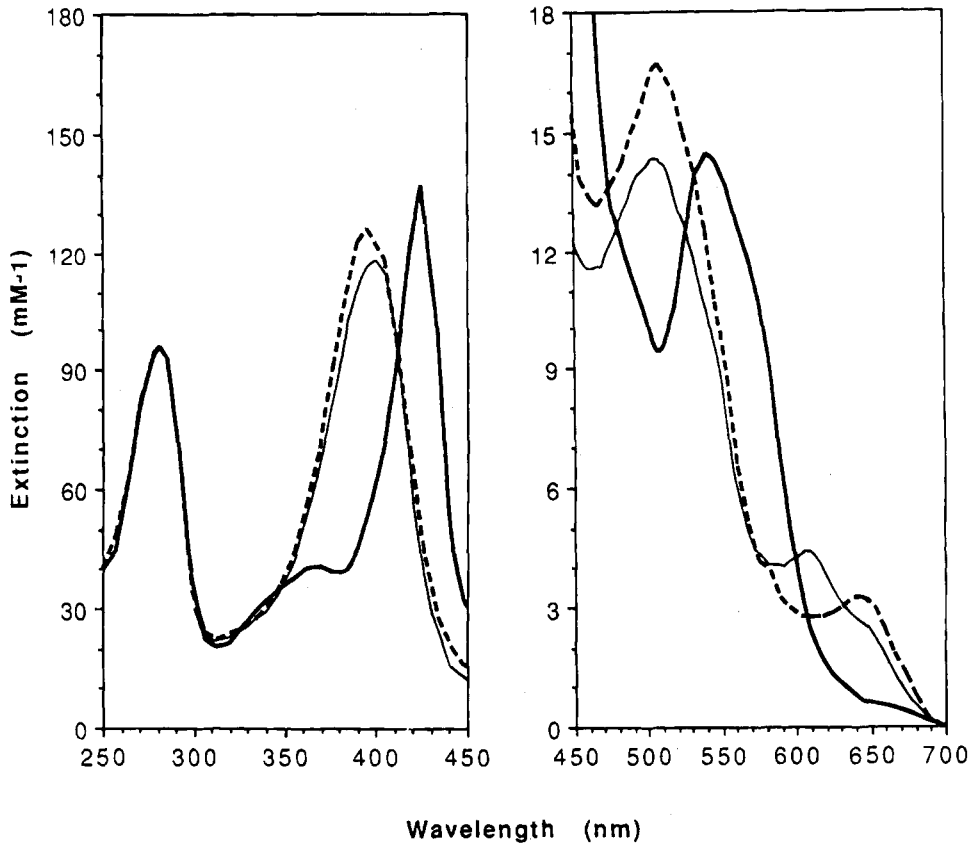

FIGURE 1: Absorption spectra of ferric FixL and its cyano and fluoro derivatives: Met-FixL (broken lines), cyano-FixL (heavy solid lines), and fluoro-FixL (thin solid lines). Protein was in 50 mM NaHepes, pH 8.0, 50 mM KCl, and 0.2 mM $MnCl_2$, at 23 °C.

was produced from a 6-h incubation of 4 μM deoxy-FixL with 2.5 mM sodium nitroprusside in phosphorylation buffer, at 23 °C, in the dark (Moriguchi et al., 1992). All of the ferrous FixL derivatives above were prepared in an anaerobic glove box. Oxy-FixL (70%) was prepared by adding sodium dithionite to a met-FixL solution containing 10 mM β-mercaptoethanol and equilibrating with air. Unless a mild reducing agent is present, ferrous FixL oxidizes to the ferric form with a half-life of 15 min in air. Spectra (350–700 nm) were recorded on a Phillips PU-8700 1-cm path length spectrophotometer at 23 °C.

Autophosphorylation. Reaction mixtures contained 2.5–5.0 μM FixL in 20 μL of 50 mM NaHepes, pH 8.0, 50 mM KCl, 0.20 mM $MnCl_2$, and [γ-^{32}P]ATP at 23 °C. Anaerobic reactions were done in a glove box under nitrogen. The reactions were started with ATP and stopped with one-third volume of gel loading buffer [4 mM EDTA, 4.0% sodium dodecyl sulfate, 0.40 M Tris-HCl, pH 6.8, 50% (w/v) glycerol, and 2% β-mercaptoethanol]; the radioactivity of [^{32}P]FixL in excised SDS–polyacrylamide gel slices was measured (Gilles-González et al., 1993). Initial rates were calculated from 1-, 2-, 3-, 4-, and 5-min time points. We define the unit rate for autophosphorylation as 1 pmol of P-FixL/nmol FixL/min. The requirements for divalent ions were determined from 5-min reactions of FixL with 0.20 mM ATP in buffer containing 0.02, 0.2, and 2 mM Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. Acetate, chloride, or sulfate salts had the same activities. The effect of elevated pH on the activity of oxidized FixL was assessed from 5-min reactions of met-FixL with 0.20 mM ATP at pH 8.1–11.2. The buffers were a mixture of 25 mM sodium bicarbonate and 25 mM sodium borate titrated with NaOH to the desired pH.

Sequence Comparisons. Protein sequences were aligned with the SIP computer program (Staden, 1982).

RESULTS

Saturation of FixL with Heme Ligands. Figure 1 shows the absorption spectra of the ferric FixL derivatives. At pH 8, met-FixL exists predominantly in a high-spin pentacoordinate form, without either H_2O or OH^- at the sixth coordination position of the iron (Gilles-González et al., 1994). The Soret absorption maxima of met-FixL and cyano-FixL are at 395 and 424 nm, respectively (Figure 1). Absorption at either of those wavelengths relative to that at the isosbestic points can be used to determine the fraction of bound cyanide. The cyanide affinity is extremely low; 50% saturation of ferric FixL occurs at 0.35 mM KCN, as compared to about 10 μM for myoglobin (Antonini & Brunori, 1971). Binding of cyanide to FixL is cooperative, with a Hill coefficient, n, of 1.7 (Figure 2). We have measured the same Hill coefficient of 1.7 for binding of cyanide to a truncated *R. meliloti* FixL, called RmFixLH, which is monomeric and contains a single heme (Gilles-González et al., 1994). Therefore this cooperativity must be caused by the binding of cyanide elsewhere in the FixL monomer rather than by interactions between the two hemes in the FixL dimer. RmFixLH has a higher affinity than *B. japonicum* FixL for all heme ligands tested so far.

Titration of 8 μM met-FixL with sodium fluoride produced an absorption peak at 608 nm. Figure 1 shows the relevant regions of the met- and fluoro-FixL spectra.

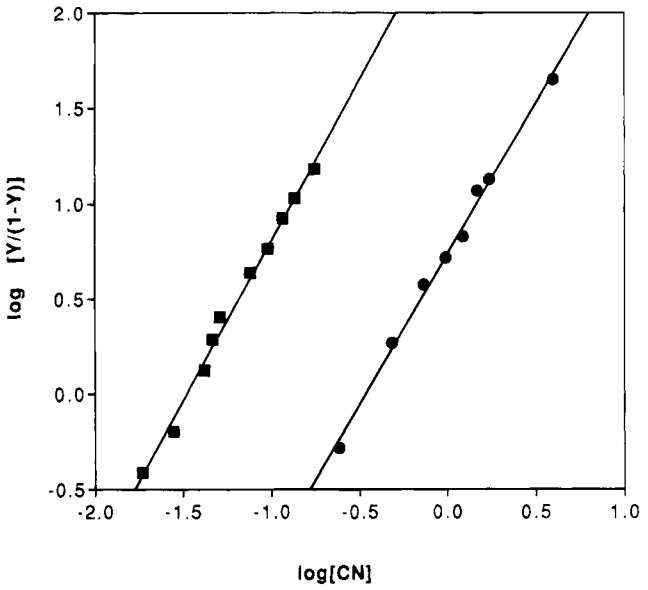

FIGURE 2: Hill plot of cyanide binding to met-FixL. The curves for met-FixL from *B. japonicum* (●) and for met-RmFixLH, the heme domain of the *R. meliloti* FixL (■), are shown. The Hill coefficient, *n*, is 1.7 in both proteins. *B. japonicum* FixL (25 μM) was titrated with 0.25−8.0 mM KCN in buffer containing 50 mM NaHepes, pH 8.0, 50 mM KCl, and 0.2 mM $MnCl_2$ at 23 °C. RmFixLH (5 μM) was titrated with 0.02−2.0 mM KCN in the same buffer.

Autophosphorylation of High- and Low-Spin FixL. The optimal conditions for autophosphorylation of the *B. japonicum* FixL are similar to those of the *R. meliloti* FixL*, including a specificity for Mn^{2+} and an insensitivity to pH over the range of 7.0−8.5 (Gilles-González & González, 1993). Between pH 8.5 and 11, the activity of met-FixL decreased 5-fold, with the midpoint for this transition occurring near pH 9.5 (data not shown). The activities measured with 0.2 mM of each of the divalent ions were as follows: Mn^{2+}, 4.8 units; Co^{2+}, 1.3 units; Mg^{2+}, 1.0 unit; Zn^{2+}, 0.1 unit. At 0.2 mM Mn^{2+}, the ferrous and ferric high-spin derivatives deoxy-, met-, and fluoro-FixL all had full phosphorylating activity of 5 ± 0.5 units, while the low-spin derivatives cyano-, oxy-, and carbonmonoxy-FixL and nitric oxide−FixL were inactive. Figure 3 shows autophosphorylation time courses for the high-spin derivatives and for partially saturated oxy and cyano derivatives. In air, ferrous FixL is 70% oxy and 30% deoxy (Gilles-González et al., 1994); this mixture has an activity of 1.4 units, about 30% of that of pure deoxy-FixL. A related FixL from *R. meliloti,* which is 15% deoxy in air, has 15% of the activity of the pure deoxy species (Gilles-González et al., 1994; Gilles-González & González, 1993). Carbon monoxide and nitric oxide inactivate deoxy-FixL (data not shown).

The cyano-FixL preparation whose autophosphorylation is shown in Figure 3 contains 90% cyano-FixL and 10% met-FixL, as measured spectroscopically; this preparation had an activity of 0.47 unit, 10% that of pure met-FixL. We have titrated preparations of met-FixL with cyanide and found their activities relative to that of ligand-free high-spin met-FixL to be the same as the fraction in that form, indicating that the low-spin cyano form is inactive (Figure 4).

Reaction of Ferrous and Ferric Unliganded FixL with ATP. The autophosphorylations of met- and deoxy-FixL, both of which are high-spin though their oxidation states

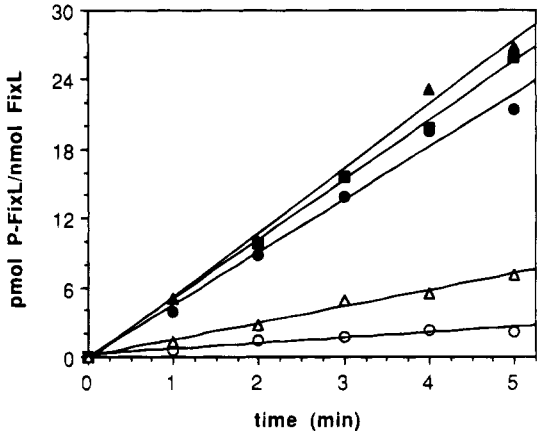

FIGURE 3: Autophosphorylation of FixL heme derivatives: time courses of autophosphorylation at saturating ATP (200 μM ATP) for met-FixL (●), deoxy-FixL (▲), fluoro-FixL (■), 30% deoxy/70% oxy FixL (△), and 10% met/90% cyano FixL (○). The 10% met/90% cyano FixL mixture was achieved by equilibrating met-FixL with 1 mM KCN. The preparations of the met-, deoxy-, fluoro-, and oxy-FixL derivatives are described under Experimental Procedures. We define 1 unit of autophosphorylation as 1 pmol of P-FixL/nmol of FixL/min.

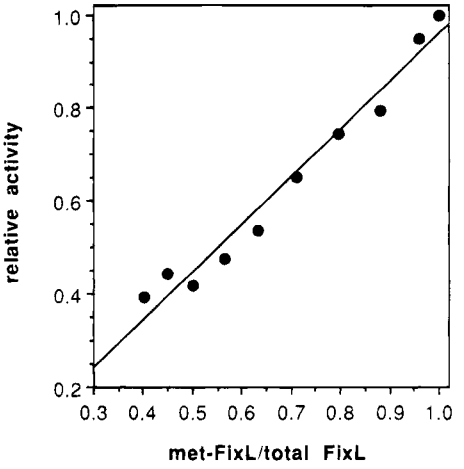

FIGURE 4: Dependence of autophosphorylation activity on the fraction of FixL in the high-spin form. We assayed the autophosphorylation activity of *B. japonicum* met-FixL (2.5 μM) in 0.05−0.45 mM KCN. "Relative activity" refers to these activities expressed as a fraction of the activity of pure met-FixL. The abscissa shows the fraction of met-FixL in the resulting met/cyano FixL mixtures. This was calculated from the cyanide concentration and the known Hill equation obtained from Figure 2.

differ, are very similar. The apparent K_m for the reactions of met-FixL and deoxy-FixL with ATP is 47 μM, and the V_{max} is 0.031 μM/min (Figure 5).

Sequence Comparisons. The heme domains of FixLs from *R. meliloti, B. japonicum,* and *Azorhizobium caulinodans* do not align with those of any known globins or any other heme proteins; there is not even a recognizable heme binding motif (Vinogradov et al., 1993). The highly conserved histidine (histidine 200 in *B. japonicum* FixL) is likely to be the proximal histidine, but there are no other conserved histidines that could be distal ones (Figure 6). The absence of a distal histidine in the FixL sequence is consistent with the ferric FixL heme iron being five-coordinated, because it lacks a hydrogen-bonding residue distal to the heme (Gilles-González et al., 1994). We have found the only protein with a definite FixL heme domain (22% identity), but not the expected kinase domain, to be the *A. caulinodans* protein

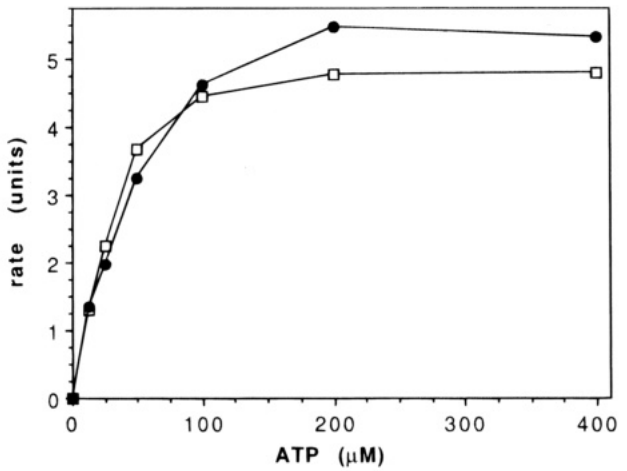

FIGURE 5: Effect of oxidation state on FixL autophosphorylation. Initial rates of autophosphorylation were measured for 5.0 μM deoxy- (●) or met-FixL (□) at varying concentrations of ATP, in a total volume of 20 μL. One unit of autophosphorylation is 1 pmol of P-FixL/nmol of FixL/min. Details of sample preparations and autophosphorylation assays are given under Experimental Procedures.

ORF1 (Pawlowski et al., 1991). This protein's function is unknown.

DISCUSSION

We have found that ferrous as well as ferric low-spin heme ligands inhibit the kinase activity of FixL in direct proportion to the fraction of total FixL bound to ligand (Figures 3 and 4). Consistent with this finding, the activity of ferric FixL is reduced dramatically above pH 8.5 (data not shown), on formation of the low-spin hydroxymet species (Gilles-González et al., 1994). The high-spin complex of ferric FixL with fluoride has full activity (Figure 3). Unliganded FixL, which is high-spin in both the ferrous and ferric states, is also fully active (Figures 3 and 5). It appears that all FixL derivatives with low-spin iron are inactive, while high-spin derivatives, whether ferrous or ferric, liganded or unliganded, have full enzymatic activity. Hence the control of the kinase domain by the heme depends solely on the spin state of the heme iron. Lack of discrimination in favor of particular oxidation states or heme ligands makes the sensor very versatile. It could sense a variety of heme ligands and even function indirectly as a redox sensor in the presence of a ligand for only one oxidation state. Virtually all heme ligands are specific for only one oxidation state, and strong ligands induce a transition from high to low spin. For example, ferrous hemoglobin undergoes a spin-state change on binding its physiological ligands oxygen and carbon monoxide, but ferric hemoglobin does not bind these ligands.

Rhizobia living under different conditions might use FixL to detect different ligands. During early nodule development FixL controls expression of the alternative oxidase that allows respiration of the bacteroids at reduced oxygen pressures (Fischer et al., 1993). FixL probably detects oxygen at this stage of transition from aerobic to microaerobic life. When nodule development is complete, oxygen pressures are held at 10 μm Hg, and all of the FixL is in the high-spin ferrous deoxy state (Appleby, 1969; Wittenberg et al., 1972). In the absence of any interfering oxygen signal, CO or NO could be sensitively and accurately detected. This could be relevant to denitrification, a process known to occur in *R. meliloti* and *B. japonicum*, which produces NO as an intermediate (Chan & Wheatcroft, 1993). The accumulation of NO from denitrification would be expected to shut off nitrogen fixation at high nitrate levels. Effectors that interact directly with the kinase or alter the affinity of the heme could provide additional regulation.

In light of the spin-state regulation of FixL, the activity of the soluble guanylyl cyclase may also be regulated through the spin state of its heme. Stone and Marletta (1994) have purified the soluble guanylyl cyclase with a high heme content for the first time. They have shown that the low-spin ligands nitric oxide and carbon monoxide enhance the activity of their new preparations of this heme enzyme. If its activity were to depend on the fraction of low-spin protein,

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RmFixL  - ARDAHLRSILDTVPDATVVSATDGTIVSFNAAAVRQ -169
BjFixL  - TRETHLRSILHTIPDAMIVIDGHGIIQLFSTAAERL -175
AcFixL  - AREAHLSSILDTVPDAMIVIDERGINQSFSITAERL -162
AcORF1  - VSEAARRAMLDTSIDAVIVADEAGAIVEFNHAAEAI -162

RmFixL  - FGYAEEEVIGQNLRILMPEPYRHEHDGYLQRYMATGEKRIIGIDRVVS -217
BjFixL  - FGWSELEAIGQNVNILMPEPDRSRHDSYISRYRTTSDPHIIGIGRIVT -223
AcFixL  - FGYSPSEVIGRNVSMLMPNPHRDQHDLYLSRYLTTGERRIIGIGRVVT -210
AcORF1  - FGHTREGVIGRPMTTIIPAHYIDRHRQGFMRHLATGENHIMRRLVEVE -210

RmFixL  - GQRKDGSTFPMKLAVGEMRSGGERFFTGFIRDLTEREESAARLEQIQAE -270
BjFixL  - GKRRDGTTFPMHLSIGEMQSGGEPYFTGFVRDLTEHQQTQARLQELQSE -276
AcFixL  - GERKDGATFPMELAVGEMHSVSGRFFTGFIRDLTERQNTEARLQELQAE -263
AcORF1  - ALRADGSVFPAELTVNEHRAGGRRLFSAFVRDISDRITSRRALERLAFT -263
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FIGURE 6: Alignment of FixL-like heme domains. Sequences of the heme binding regions of FixL from *R. meliloti* (RmFixL), *B. japonicum* (BjFixL), and *A. caulinodans* (AcFixL) are shown. A highly homologous sequence from an *A. caulinodans* protein of unknown function (AcORF1) is also shown.

then the ferrous carbonmonoxy and nitric oxide forms should have activities comparable to that of the ferric cyano form. In that case, the control of heme-enzyme activity by spin state may be widespread.

In 1965, Monod et al. proposed that feedback inhibition of oligomeric enzymes operated through an equilibrium between relaxed (R) and tense (T) structures. In T structures, conformational changes required for activity are hindered by additional bonds between the subunits of the oligomers, while in the R structure, rupture of these bonds allows full activity. In hemoglobin, the T to R transition of the tetramer is triggered by a movement of the heme iron 0.4 Å into the plane of the porphyrin nitrogens on transition from high to low spin (Perutz, 1970, 1989; Messana et al., 1978; Perutz et al., 1978). The R state of hemoglobin lacks the salt bridges between the $\alpha 1$ and $\beta 2$ subunits that are present in the T state (Perutz, 1970). These salt bridges lower oxygen affinity by hindering the changes in tertiary structure needed to accommodate the high- to low-spin transition. We propose that the transition from low to high spin in dimeric FixL results in a transition from a t to an r form, but instead of altering the affinity of the hemes, this transition in FixL activates the kinase domains.

Ninfa and her colleagues (1993) recently demonstrated cross-phosphorylation of subunits in the closely related dimeric sensor kinase NtrB. They have shown that one monomer first binds ATP, and then the γ-phosphate of the ATP is transferred to a phosphorylation site on the other monomer distinct from its ATP binding site. Control of phosphorylation in a dimer could occur in three ways in response to a ligand-induced t to r transition: (1) interference with ATP binding, (2) interference with the phosphorylation site, or (3) prevention of proper relative orientation of the monomers for phosphoryl transfer. Mechanism 3 would lead to a nonlinear dependence of activity on saturation with ligand, since binding of ligand to either monomer would inactivate both monomers. In FixL, we can measure ligand binding from absorption spectra independently of activity and can rule out mechanism 3 on the basis of Figure 5. The very high homology (>20% identity) among sensor kinases makes it likely that despite the immense diversity of sensor domains and the molecules they detect, the mechanism by which they control their kinase domains will be similar [reviewed in Parkinson and Kofoid (1992)]. Hexokinase and many other kinases have a "nutcracker" structure made of two lobes, one that binds the substrate and another that binds ATP (Anderson & Steitz, 1975). On activation, the two lobes close. Conceivably, the spin state of the heme iron in FixL might control the opening and closing of such a nutcracker.

FixL heme domains are not limited to FixL or even to proteins belonging to the two-component sensor kinase family. ORF1 is a 736 amino acid protein without a homologous kinase domain or any recognizable function (Pawlowski et al., 1991). We found a region of ORF1 that is 22% identical to the heme binding domain of FixL (Figure 6). This suggests that there are other heme domains, related to FixL, functioning as sensor modules in entirely different molecular contexts.

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