MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Sept. 2004, p. 453–473 Vol. 68, No. 3
1092-2172/04/$08.00+0 DOI: 10.1128/MMBR.68.3.453–473.2004

CO-Sensing Mechanisms

Gary P. Roberts,* Hwan Youn, and Robert L. Kerby

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin

INTRODUCTION

Sources and Biological Impacts of CO

Under ambient conditions, carbon monoxide (CO) is a colorless and odorless gas long associated with incomplete combustion and best known for its toxicity. Vivid descriptions of deaths attributed to "fuel gas" leaks were common in the 19th century, since CO comprised 5 to 40% of this fuel (85). Evidence of its more pervasive and subtle biological properties has been revealed during the past century of discoveries. We know that CO serves (i) as a substrate for microbial growth, (ii) as a central metabolite in the anaerobic carbon cycle and component of a novel anabolic pathway, (iii) as an intrinsic metal ligand in enzymes, (iv) as a gaseous signal in microbes and mammals, and (v) as a speculative component in an early mode of metabolism and the origin of life (89).

CO is a ubiquitous and globally increasing atmospheric pollutant (150 ppb) largely generated by the burning of fuels and biomass, with apparently minor contributions from numerous biological systems (23, 78, 132), which continue to be discovered (see, e.g., reference 25). Its removal occurs primarily through photochemical oxidation, along with significant degree of microbial utilization (23, 90). In humans, exposure to urban CO levels (ca. 100 ppm) and tobacco smoke may result in a variety of symptoms that are easily mistaken for viral illnesses (1, 134), while acute toxicity occurs at higher exposure, typically via the accumulation of combustion products in confined spaces with improper ventilation. As a result, some 2,000 non-fire-related CO-poisoning deaths occur annually in the United States (92). Human toxicity is attributed to the high affinity of CO for iron such that the oxygen capacity of hemoglobin is reduced, and the gas is broadly inhibitory for heme proteins and nonheme iron enzymes including hydrogenase (2, 45, 106) and nitrogenase (20, 83, 106). In light of its heme affinity, it is a biological irony that the primary endogenous source of CO in mammals is heme degradation catalyzed by multiple heme oxygenase activities in the cell. These heme oxygenases are differentially expressed and regulated, and they produce micromolar levels of CO in cell cultures (12). On average, humans produce 0.4 ml of CO per h. This endogenous CO production in mammals highlights the importance of the discrimination against CO by hemoglobin. Without such discrimination, endogenous CO production alone would result in 20% CO-bound hemeproteins (22).

* Corresponding author. Mailing address: Department of Bacteriology, 420 Henry Mall, University of Wisconsin—Madison, Madison, WI 53706. Phone: (608) 262-3567. Fax: (608) 262-9865. E-mail: groberts@bact.wisc.edu.

Biological Utilization of CO

Beginning a century ago, evidence of microbial growth in the presence of CO has steadily accumulated. As thoroughly reviewed by Uffen (132) and Kim and Hegeman (78), studies through 1980 demonstrated the utilization of CO by diverse aerobic ("carboxidotrophic") and the anaerobic microbes, both as a catabolic substrate in some and as an adventitious substrate—the result of low enzyme specificity—in others. Yagi deliberately investigated CO oxidation in an extract of *Desulfovibrio desulfuricans* (143), but chance played a role in the discovery of CO oxidation by extracts of the saccharolytic *Clostridium pasteurianum* when the gas was used as a hydrogenase inhibitor (123). Starting in the mid-1980s, considerable research efforts changed this CO dehydrogenase (CODH) activity from a metabolic curiosity to a central intermediate in anaerobic catabolism and anabolism and defined two general microbial systems, one aerobic and one anaerobic, that utilize CO. Extensive reviews are available (34, 39, 40, 87, 91, 104, 142). In another biological irony, in light of its historical use as an inhibitor, recent results indicate that CO is an intrinsic iron ligand in the Fe- and Ni/Fe-containing hydrogenases (2, 45, 106), as well as in CODH itself (35, 61).

Diverse aerobic carboxidotrophs express heterotrimeric Mo-containing enzymes that couple CO oxidation ($CO + H_2O \rightleftharpoons CO_2 + 2e^- + 2H^+$) to CO-insensitive respiration (87, 91). These enzymes characteristically demonstrate high affinity for CO, in part reflecting its reduced solubility at the thermophilic growth temperatures of several carboxidotrophs, and the oxidation is typically coupled to CO_2 fixation via the Calvin-Benson-Bassham reductive pentose phosphate cycle. CO-dependent expression has been shown, although the mechanism of this regulation remains undefined (113). Anaerobic or anaerobically cultivated members of the *Archaea* (methanogenic and sulfate reducing) and *Bacteria* (sulfate reducing, acetogenic, hydrogenogenic, and phototrophic) express (sometimes multiple) homodimeric, heterodimeric, and heteropentameric, and heteropentameric enzymes that catalyze reversible CO oxidation. These possess a Ni-containing C site, an activity readily assessed when coupled to dye reduction. Sequence analyses show remarkable protein conservation despite the diverse lineages (32, 74). In addition, the heteromeric enzymes catalyze an acetyl coenzyme A (acetyl-CoA) synthase (ACS) activity at the metallocluster A site, also containing Ni, that interconverts acetyl-CoA with CO, a cofactor-bound methyl group, and coA. The anabolic formation of acetyl-CoA represents the identifying feature of a widespread mechanism of carbon fixation termed the Wood-Ljungdahl pathway and has been suggested as a primordial anabolism (64). In addition, CODH-ACS catalyzes the reverse process during the anaerobic catabolism of acetate (34, 39, 40, 52, 104, 142).

It is important to note that (i) the CODH-ACS-catalyzed one- and two-carbon interconversion is a central function in anaerobic metabolism in which CO is a sequestered intermediate in the reaction and (ii) as a fundamental anabolic and/or catabolic anaerobic process, the bifunctional Ni-containing enzymes are expressed independently of exogenous CO (see. e.g., references 7 and 72). In contrast, expression of the monofunctional CODH enzymes can readily be associated with environmental CO: the anaerobic *Carboxydothermus hydrogenoformans* (Ni-containing CODH) was obtained from a volcanic vent (121), while the aerobic *Streptomyces thermoautotrophicus* (Mo-containing CODH) was isolated from soil covering mounds of burning charcoal (110).

Structures of both the Mo- and Ni-containing enzymes have been published recently. The carboxydotrophic enzymes isolated from *Oligotropha carboxidovorans* and *Hydrogenophaga pseudoflava* display hydrophilic and hydrophobic channels to the Mo- and Cu-containing reaction center and FeS centers appropriately spaced for electron transfer (30, 31, 49, 55). Published structures (26, 33) of the bifunctional enzyme from the anaerobe *Moorella thermoacetica* (formerly *Clostridium thermoaceticum*) also display a complement of FeS centers for electron transfer, as well as the A and C Ni-containing reaction centers connected by a 70-Å hydrophobic channel through which CO transits. The evident channeling, also indicated experimentally (86, 115), confirms the role of CO as a central metabolic intermediate despite its modest solubility and environmental paucity. Finally, structures of monofunctional Ni-containing enzymes from the thermophilic *C. hydrogenoformans* and the photosynthetic *Rhodospirillum rubrum* have been reported (32, 35). These are structurally similar to the β-subunit of the *M. thermoacetica* enzyme and contain the CO-oxidizing C center. In *R. rubrum*, carbon fixation occurs via the Calvin-Benson-Bassham cycle or alternative mechanisms but not via acetyl-CoA synthesis (69).

Anaerobic, CO-dependent catabolism by phototrophs was first described in 1968 (63) and subsequently elaborated for a strain of *Rhodopseudomonas* (now *Rubrivivax gelatinosis*) (131) and *R. rubrum* (13, 37, 74, 132). The *R. rubrum* process, the subject of considerable studies in the laboratory of Paul Ludden, depends on the CO-induced, anaerobic expression of a monofunctional Ni-CODH, a CO-insensitive hydrogenase (41), associated electron transfer (42), and Ni-mobilization (68, 73, 138) components. Eleven identified genes, designated *coo* (for "CO oxidation"), are organized into two regulated transcripts. One encodes the hydrogenase subunits and associated components (*cooMKLXUH*), and the other encodes CODH (*cooS* gene product), an unusual FeS protein, and components for Ni storage and insertion (*cooFSCTJ*). Remarkably similar proteins (and genetic arrangement) are found in *C. hydrogenoformans* (118). In *R. rubrum*, energy derived from the thermodynamically marginal process ($CO + H_2O - CO_2 + H_2$, $\Delta G^{o\prime} = -20$ kJ/mol) depends on vectoral proton translocation, possibly by CooU, CooM, CooK (42), or CooX (4). Genes for similar CODH enzymes and overlapping sets of the auxiliary functions have been found in the sulfate-reducing *Desulfovibrio vulgaris* Hildenborough (137), *Desulfovibrio desulfuricans* (wherein Yagi first detected CODH activity [143]), and the aerobic nitrogen-fixing *Azotobacter vinelandii*. As described below, all of these organisms contain a gene homologous to *cooA*, whose product in *R. rubrum* senses environmental CO and initiates transcription of the CO-oxidizing/H^+-reducing system. Descriptions of studies of CooA of *R. rubrum* form a major part of this review because of the following broad biological implications: (i) it is the clearest example of biological CO signal reception, a phenomenon recently proposed for mammalian systems as well (12), (ii) it is a prototypical heme-based sensor, and (iii) it serves as a model for the mechanism of action of other members of the CRP/FNR (cyclicAMP

[cAMP] receptor protein/ fumarate nitrate reductase regulator) family of transcriptional regulators.

BIOLOGICAL CO SENSORS

General Features of CO Binding and Selectivity

The special affinity of CO for metal atoms makes it an inhibitor of a number of metalloproteins, and all the CO sensors discussed below also contain metals. All CO sensors thus far described rely on the specific metal center found in hemes, and it is somewhat surprising that sensors with metal-sulfur centers have not yet been found. Indeed, nonheme proteins have been identified as sensors for oxygen, such as FNR (77) and SoxR (102); for H_2, such as HoxAJ (10); and for NO, such as NnrR (127); a short review of sensors involved in NO metabolism has recently been published (151).

The existence of heme-containing CO sensors is itself unsurprising, since CO can bind to virtually all heme-containing proteins. This property means that all heme-containing proteins are possible candidates for being biological CO sensors even though they are known to have other obvious physiological gaseous ligands. CooA, the only CO sensor demonstrated to be physiologically relevant, and other candidate CO sensors have similar general properties to other heme-based sensors. As a group, heme-based sensors commonly exploit histidine as a proximal ligand. Typically, this Fe-His bond is weak, as evidenced by low Fe-His stretching frequency in the resonance Raman spectrum (126, 129), but the functional role of this weak bond is unclear. On the other hand, each heme-based sensor possesses a unique heme environment, which affects its specificity for different small-molecule ligands. There are three levels of ligand specificity in heme-based sensors. The first is at the level of binding, which is important for heme-based O_2 sensors because the natural affinity for O_2 is lower than that for CO by a factor of ~20,000. In hemoglobin and myoglobin, the ratio is reduced to 25 to 200 (119) by exploiting H-bonding between the distal histidine side chain and O_2 ligand. Nitrophorins, NO transporters found in the saliva of the blood-feeding insect *Rhodnius prolixus*, use the unique NO binding to Fe(III) heme for ligand discrimination, to which state CO or CO_2 hardly binds (18). The second level of ligand specificity involves the coordination property of the ligand. This level is useful for NO sensors since NO uniquely exerts a strong *trans* effect, occasionally weakening the *trans*-ligand bond so much that a five-coordinate NO adduct results. This is important for the selectivity of soluble guanylate cyclase (sGC) for NO, as described below. The third level of selectivity is through allostery, which is unique in each heme-based sensor and represents the conformational change that the sensor undergoes in response to ligand binding. For a given sensor, the ligand specificity is likely to be provided by a combination of these three levels. For each of the following examples of sensors capable of binding CO, we briefly address what is known about the molecular basis for ligand discrimination.

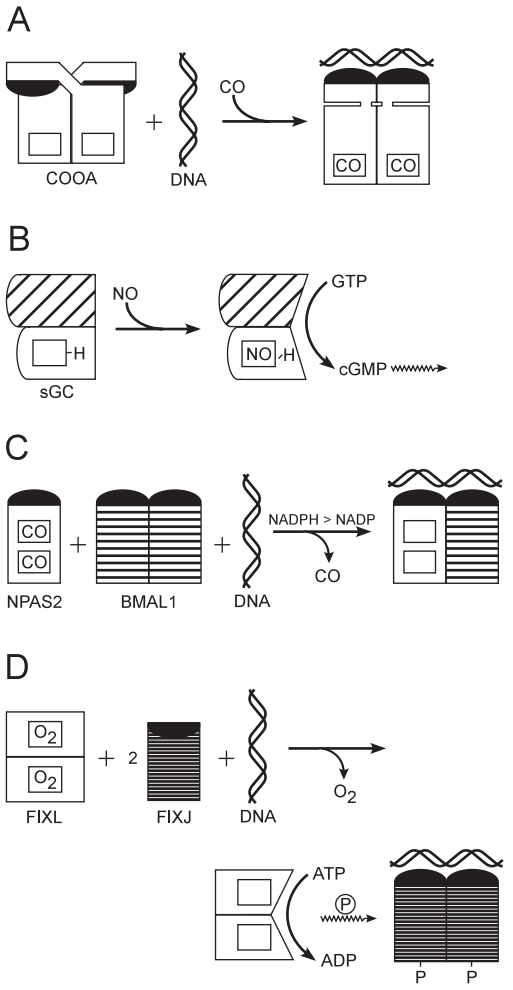

FIG. 1. The general behaviors of CooA, sGC, NPAS, and FixL in response to their effectors. In all cases, the relevant heme pockets are depicted as boxes. (A) In the absence of CO, the CooA homodimer has its DNA-binding surfaces (shaded black) buried away from the solvent. On CO binding, there is a significant rearrangement of a portion of each monomer that allows the DNA-binding surfaces to bind specific DNA sequences. The DNA-bound CooA then interacts with RNA polymerase to allow gene transcription. (B) sGC exists as a heterodimer that is inactive without NO. NO binding to the heme displaces an endogenous histidine ligand (−H), which triggers a conformational change. The active conformation of sGC synthesizes cGMP, an important signal molecule. (C) NPAS2 is only recently described and poorly understood. In the presence of small molecules bound to the heme, the protein exists as an inactive monomer, but the absence of such small molecules leads to the formation of an active heterodimer with another protein, BMAL1, which is able to bind DNA and activate transcription. (D) FixL acts as a homodimer that is inactive when O_2 is bound to the hemes. In the absence of O_2, FixL undergoes a conformational change that causes autophosphorylation and subsequent transfer of that phosphate to FixJ. Phosphorylated FixJ binds DNA and activates transcription of anaerobically expressed genes.

CooA and CooA Homologs

The best understood CO-sensor is CooA, which regulates the CO oxidation system of *R. rubrum* (111). CooA is a homodimeric heme-containing protein that is a specific CO sensor in *R. rubrum* (5, 58, 108, 117, 146) (Fig. 1A). As mentioned above, the *coo* gene products are organized into two operons, whose expression appears to be controlled exclusively by CooA. The only substrate of the *coo* gene products is CO, and so the CO specificity of CooA is biologically important. CooA

is also a redox sensor, which is important because the regulated CODH only catalytically active only at potentials below −300 mV (60). The basis of this redox-sensing is that CO binds only to the reduced form of the CooA heme, and the midpoint potential of this reduction is approximately −300 mV as well (94). Because of the wealth of knowledge about the behavior of CooA, especially with respect to its CO specificity, its description occupies a large part of this review and is only briefly summarized here.

CooA is a distantly related homolog of the well-studied transcriptional activator CRP of *Escherichia coli* (also known as CAP, for "catabolite activator protein"), which has cAMP as its effector molecule. Comparisons between the structures of the two proteins have allowed testable hypotheses about the mechanism of their activation by their respective small molecules (81).

CooA of *R. rubrum* is the prototype of a family of related proteins from a wide variety of bacteria that are apparently involved in CO sensing (147). This claim is based on the following arguments. (i) The CooA homologs all have a deletion of eight amino acids (with respect to the CRP sequence) that appears to provide space for the heme in CooA of *R. rubrum*. A number of other residues already shown to be critical for the functionality of CooA of *R. rubrum* are also conserved in the homologs. (ii) All of these homologs are found in genomes where there is also a gene for a CO dehydrogenase that is homologous to the NiFe CODH of *R. rubrum*, whose expression is regulated by CooA in that organism. (iii) The genes for six of these CooA homologs have been cloned in *E. coli*, and four allowed CO-dependent gene expression in the *E. coli* reporter system used for analysis of CooA of *R. rubrum* (147). (iv) The F helices of all the homologs, which serve to make specific DNA sequence contacts, are similar to each other, and there are appropriate palindromic sequence 5′ of the genes for the CO dehydrogenases in each organism, although the actual biological function of these palindromes has not been experimentally demonstrated. Further discussion of the implications of these homologs for the general behavior of the CooA family is presented in the various appropriate sections that discuss CooA function in detail.

Soluble Guanylate Cyclase

sGC is expressed in the cytoplasm of almost all mammalian cells and mediates a wide range of important physiological functions (21) (Fig. 1B). Vertebrate sGC is a heterodimeric (α/β) heme protein that senses NO (70, 120). When NO binds to the heme of sGC, its activity in converting GTP to cGMP is enhanced by several hundredfold. The enzymatic product, cGMP, is a secondary messenger that plays a pivotal role in a variety of physiological processes including vasodilation and neuronal signal transduction (21).

The structure of sGC is unknown, but its catalytic domain is thought to be composed of the C-terminal regions of the both subunits, which have sequence homology to regions in the particulate sGC and the adenylate cyclases. While both subunits are essential for catalytic activity, the N-terminal region of the β subunit alone binds the heme prosthetic group (43). Not surprisingly, the presence of the heme prosthetic group is required for activation of sGC by NO (65) and NO has been shown to trigger a conformational change in sGC through the cleavage of the proximal histidine ligand, His105, resulting in five-coordinate high-spin NO adduct (150). The study of sGC by using a series of metalloporphyrins supports the hypothesis that it is the cleavage of the His-Fe bond that is critical (17, 28). For example, Mn(II)-containing protoporphyrin IX-reconstituted sGC binds NO but is not active, consistent with the role of the retention of the His-Fe bond. On the other hand, Ni(II)- or Cu(II)-containing protoporphyrin IX-containing sGC is active without NO. This latter result is easily rationalized by the fact that Ni(II) or Cu(II) protoporphyrin IX favors four-coordinate geometry, and therefore these reconstituted sGC variants lack the His-Fe bond. Probably by a similar mechanism, sGC with a free-base protoporphyrin IX, lacking an iron and therefore incapable of forming the His-Fe bond, is active. However, the observation that heme-free sGC is not active indicates that cleavage of the His-Fe bond is not sufficient and that the heme vicinity also plays a crucial role in the activation mechanism of sGC.

Recently, CO has been shown to inhibit platelet aggregation and promote the relaxation of vascular smooth muscle (15, 133), which are activities normally attributed to the action of NO on sGC. Consistent with the hypothesis that this CO activity reflects the activation of sGC, these CO activities have been shown to be cGMP dependent (15, 105). It has been known for some time that CO can enhance sGC activity in vitro, although its fourfold effect is much lower than that of NO and seems inconsistent with the degree of physiological response seen. Indeed, since CO binding to sGC leads to a six-coordinate low-spin heme adduct, with an intact His-Fe bond, the activation mechanism of CO must be different from that of NO. Importantly, however, the presence of YC-1 [3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole], a small synthetic molecule that has been found to enhance the CO effect in vitro, allows activation by CO to reach the same level as that of NO-stimulated sGC (44). The molecular basis for the stimulation mechanism of YC-1 to the CO-bound sGC is unclear, but YC-1 apparently does not force the cleavage of the Fe-His bond (75), as might be expected if it also led to a five-coordinate adduct. The question remains whether CO is a physiological ligand of sGC and if there is a natural product in vivo that mimics the stimulation caused by YC-1 in vitro. While there is no evidence available for the latter question, there are strongly suggestive data in support of the former. First, CO-induced vasorelaxation can be blocked by inhibiting sGC activity (46), consistent with a direct role in vivo for CO and sGC. Second, there is the striking evidence that heme oxygenase, the source of CO in nerve cells, colocalizes with sGC in cells with little or no nitric oxide synthase expression, supporting a link between CO and sGC (66, 135, 136). While the possible role of sGC in mammalian CO sensing is tantalizing but unproven, there is little doubt that some mammalian CO sensor must exist.

Neuronal PAS Domain 2

Neuronal PAS domain 2 (NPAS2) is a recently described mammalian transcription factor that, together with BMAL1, binds DNA as an obligate heterodimer. It has been implicated in regulation of the circadian rhythm (11, 29) (Fig. 1C). The NPAS2 monomer contains two hemes as CO-binding sites,

which are both six-coordinate in their resting states. The functional consequence of CO binding to NAPS2 is the accumulation of BMAL1 homodimers at the expense of NPAS2-BMAL1 heterodimers in vitro (29). However, some curiosities remain to be explained. First, Dioum et al.(29) reported that NPAS2 derivatives failed to bind NO effectively, which is highly unusual in heme-containing proteins. Second, the same authors reported that O_2 reacted irreversibly with NPAS2, suggesting that the O_2-bound adduct was as functional as the CO-bound adduct for the activity examined. Finally, NAPS2 is under the regulation of the intracellular NADP/NADPH ratio even without CO. It remains to be determined if CO is the physiological ligand of NPAS2 in vivo.

FixL

The FixL proteins found in the rhizobia sense O_2 and regulate the expression of the genes associated with nitrogen fixation as a function of O_2 pressure (48) (Fig. 1D). Although not demonstrated to be a physiologically significant CO sensor, FixL is addressed in this review for several reasons. First, as detailed below, the purified protein shows a significant response to CO. Second, the response of FixL to a variety of small-molecule ligands serves as a useful counterpoint to the extreme ligand specificity of CooA. Finally, because CO is a potent inhibitor of nitrogenase (20, 83), the notion of a physiologically significant response by FixL to CO is not preposterous.

In the absence of O_2, FixL is autophosphorylated at an invariant histidine residue and the phosphoryl group is transferred to FixJ, leading to an enhancement in transcriptional activity. Under aerobic conditions, O_2 binding to the heme domain inhibits the histidine kinase activity in the C-terminal domain by 15-fold. The heme-binding domain of FixL proteins is a PAS domain (an acronym for the proteins with this domain, Per/Amt/Sim) (38, 122), a sequentially and structurally conserved motif that commonly serves as the sensor module of two-component signal transducers. In addition to FixL, two types of O_2-sensing phosphodiesterase, EcDos (27) and AxPDEA1 (19), contain PAS heme sensors.

Although the cognate physiological effector of FixL is certainly O_2, there is substantial disagreement in the literature about both the degree and the basis of ligand specificity. Many of the challenges in addressing this issue have recently been described (36, 128). The first issue is that many of the original hypothesis about ligand specificity were based on structures of only a portion of FixL and thus missed any effects of the rest of this protein or of FixJ, its regulatory partner. The second concern is that there are a number of biochemical properties of the protein that are altered in a variety of ways by the binding of different small-molecule effectors, so that the physiologically significant biochemical response remains unclear. Finally, the situation is complicated by the fact that FixL has been extensively studied from two different organisms, *Bradyrhizobium japonicum* and *Sinorhizobium meliloti*. However, the assays and the data sets for the two proteins are different, and it appears that their biochemical properties might be different as well. In the autophosphorylation assay, FixL of *S. meliloti* shows a broad ligand specificity in vitro (128). CO induces a conformational change, resulting in a 5-fold decrease of autophosphorylation activity of FixL, while NO causes a 2-fold decrease, CN^- causes a 15-fold decrease, and imidazole causes a >75-fold decrease. With FixL of *B. japonicum*, the more relevant "turnover" assay, which measures the phosphorylation of FixJ, shows rather more selectivity toward O_2.

Over the past few years, several hypotheses for activation, including the selectivity toward O_2, have been proposed. The first was the so-called spin-state hypothesis (47, 128), which was based on the observation that high-spin forms of FixL [Fe(III), Fe(II), and fluoro-FixL] have the same autophosphorylation activity and that low-spin forms inhibit the activity of FixL. Flattening of the heme induced by a switch of the iron atom from high spin to low spin by these ligands seemed to inactivate FixL reversibly. However, it now appears that not all low-spin forms are inhibited to the same degree, which is difficult to explain by this model, nor does it explain some recent mutational results (36). An alternative model is the "loop displacement hypothesis" which suggests that the critical element in inactivation is movement of a particular loop in the protein, termed the FG loop. While this model is attractive, the exact mechanism by which ligand binding effects this displacement remains obscure (36), although Arg220 (discussed below) probably plays a role.

The possible role of Arg220 as the trigger for the conformational change in *B. japonicum* FixL is based mainly on X-ray crystal structures. The heme-binding domains of most of the *B. japonicum* FixL structures (free or liganded form) have been solved, although these have not been in the context of the rest of the protein (50, 51, 56, 67) (Fig. 2). The binding of strong heme ligands changes the heme planarity in FixL and probably weakens the salt bridge between Arg220 and heme propionate 7 which is found in met-FixL of *B. japonicum*. The released Arg220 can move into the heme pocket and serve as a steric barrier that stabilizes the inactive FixL conformation. The greater sensitivity of FixL to O_2 and CN^- than that of other ligands can be explained by this model, since O_2 and CN^- are capable of holding the released Arg220 in the heme pocket. Nonetheless, another element must be responsible for the inhibition of the phosphorylation activity in CO-, NO-, and imidazole-bound forms of FixL, because in those structures, Arg220 movement into the pocket has not been observed (67).

Finally O_2, unlike CO and NO, has been shown to create a disulfide bond in *S. meliloti* FixL at Cys301 in the homodimer (3), thereby potentially providing another level of ligand discrimination in favor of O_2 in that organism. However, such residues are not conserved in other FixL proteins, suggesting that this mechanism is not a general one.

One might wonder why FixL shows broad ligand specificity, although it might reflect an inherently stronger affinity of free heme for NO or CO than for O_2. This challenge of competition by physiologically inappropriate small molecules is partly overcome in myoglobin through the use of polar distal heme pocket residues (most importantly a histidine residue) which preferentially stabilize O_2 over CO. However, a similar mechanism does not appear to be employed by FixL, since its distal heme pocket is composed of highly hydrophobic amino acids (Ile209, Leu230, and Val232 in *S. meliloti* FixL; Ile215, Leu236, and Ile238 in *B. japonicum* FixL). The uncertainties about the role of CO in FixL function will probably be resolved in the near future.

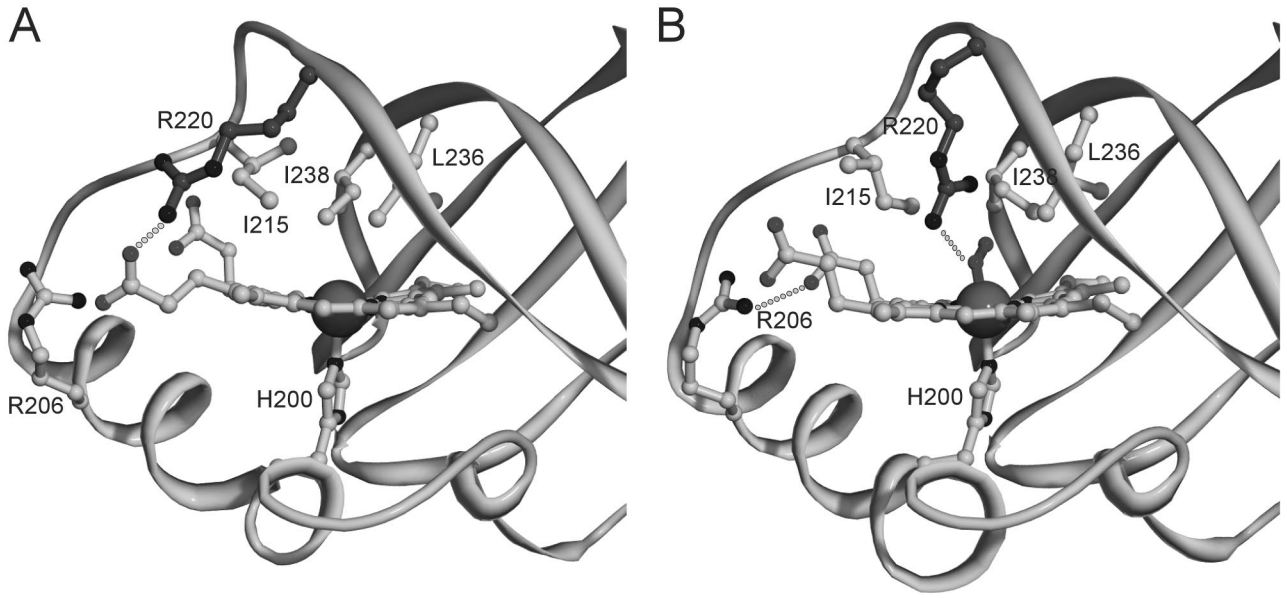

FIG. 2. Current model for the behavior of the heme vicinity of FixL in response to O_2 binding. (A) Active ferrous FixL (Protein Data Bank no. 1LSW), where the heme is depicted at the center, with the Fe atom as the dark sphere. H200 is the endogenous ligand in this five-coordinate form. (B) On O_2 binding (Protein Data Bank no. 1DP6), there is a slight movement of the Fe atom with respect to the heme but a substantial movement of heme pocket residues. The movement of R220 (darkened in the figure) is thought to be particularly significant and results in a repositioning of the heme proprionates with respect to other residues in the heme pocket.

Other Potential CO Sensors

Carboxidotrophic bacteria are aerobic chemolithoautotrophs characterized by the utilization of CO as the sole source of carbon and energy. They are taxonomically diverse bacteria, encompassing more than 15 described species in eight genera (88). The *O. carboxidovorans* CODH central to this CO utilization is an O_2-stable, molybdenum-iron-sulfur-flavin hydroxylase (53). The CODH structural genes are flanked by nine accessory genes arranged as the *cox* gene cluster (*coxB CMSLDEFGHIK*) (113). Most of the deduced products of the *cox* genes of *O. carboxidovorans* have counterparts in the gene clusters of *Pseudomonas thermocarboxydovorans*, *Hydrogenophaga pseudoflava*, *B. japonicum*, and *Mycobacterium tuberculosis*. Importantly, the *cox* genes are specifically and coordinately transcribed under chemolithoautotrophic conditions in the presence of CO as the carbon and energy source (113). While no CO sensors have yet been identified in this group of organisms, they would be expected to be unlike CooA, because these organisms must express their genes aerobically but CO binds only to the Fe(II) form of CooA. Consistent with this view, no *cooA* homologs are apparent in the genome sequences that are available for these organisms.

sGC has been described as a possible CO sensor, but other eukaryotic CO sensors might exist. There is emerging evidence suggesting that at least some CO effects are mediated through a cGMP-independent, mitogen-activated protein kinase pathway. Anti-inflammatory effects and the antiapoptotic action of CO are good examples (14, 97). The precise mechanism by which CO might activate the kinases remains to be elucidated, and the target protein of CO is not yet identified, but further studies might lead to the identification of eukaryotic CO sensors.

COOA AS A CO SENSOR AND RESPONDER

CooA of *R. rubrum* is not only the best understood heme-containing sensor but also highly specific in its response to CO as an effector. As discussed below, CO binding to the reduced heme of CooA causes a substantial conformational change in the protein that enables it to bind specific DNA sequences and thereby activate transcription. We first give some background about the activity and structural features of CooA and then address what is known about CO binding and its response.

CooA Activity Assays

The role of CooA in the cell is to activate the expression of 11 additional genes whose products are involved in CO oxidation when CO is present under reducing conditions. It performs this activation by binding to palindromic sites on the DNA that are at the 5′ ends of operons whose products oxidize CO. Immediately adjacent to the CooA-binding sites are weak promoters, and, because of protein-protein interactions between the bound CooA and RNA polymerase, the affinity of the polymerase for those promoters is increased and transcription initiation can take place (see below).

The sequences bound by CooA are reminiscent of those bound by CRP and FNR, which is consistent with the relatively high similarity among the F helices of these proteins (see below), which make the specific base contacts. For proper biological regulation of expression, the affinity of the binding site for the activator should be such that there is very little occupancy of the site in the absence of the effector but very high occupancy in the presence of the effector.

CooA has been routinely examined for its activity in vivo, using reporter systems that measure the ability of CooA to

bind a specific DNA sequence and then properly interact with RNA polymerase to activate transcription and produce a product that can be assayed. When analyzing CooA activity in *R. rubrum*, the assay has typically been the activity of the CODH itself, but this is somewhat indirect, since that activity is a reflection of not only gene expression but also CODH maturation (73, 138). There are several technical advantages in using an *E. coli* reporter strain with *lacZ* fused to one of the two normal CooA-responsive promoters from *R. rubrum*. When *cooA* is expressed from a plasmid, this strain expresses very low β-galactosidase activity unless the cells are anaerobic and exposed to CO. Such an assay shows a linear response over a certain range of CooA activity, because maximal β-galactosidase activity requires only that the CooA-binding site upstream of *lacZ* be saturated (71). Significant differences in the fraction of the CooA population in the active form can therefore be missed unless total CooA levels are tuned through regulation of the *cooA* promoter.

While more time-consuming, an in vitro assay of DNA binding by CooA has significant advantages. The most readily interpretable assay involves fluorescence anisotropy, in which a fluorescently tagged DNA fragment containing the CooA binding site is incubated with purified CooA (125). This assay can provide a K_d for any tested variant in the presence and absence of effector. It measures only DNA affinity, rather than the complex combination of DNA affinity and affinity for RNA polymerase that is measured in vivo. Other assays such as gel shifts (D. Shelver and G. P. Roberts, unpublished data), footprinting (117), and in vitro transcription assays (59) have also been successfully applied to CooA.

Insights into the Activation Mechanism from a Structural Comparison of CooA and CRP

To understand the mechanism by which CO activates CooA, and in particular the basis for the specificity of CooA for CO, it is necessary to know the structure of both the inactive (CO-free) and active (CO-bound) forms of the protein. To this point, however, only the reduced, effector-free form of CooA has been solved structurally (Fig. 3B) (81). The most striking initial feature of the CooA structure is its asymmetry, which is presumably an artifact of crystal packing. Because the "folded-down" monomer (form A in Fig. 3B) makes other contacts in the crystal lattice, it has been proposed that form B might be more representative of CooA in solution (81). Although the structure of the active form of CooA is unknown, the analysis of a homolog, CRP, has been highly informative. CRP is an extremely well-studied transcriptional factor of *E. coli* that activates the expression of genes encoding the utilization of poor carbon sources in response to cAMP binding. The results of several structures of active (cAMP-bound) CRP have been published (98, 100, 139). As depicted in Fig. 3A, the protein is a dimer, with each monomer consisting of two domains: an effector-binding domain linked by a hinge region to a DNA-binding domain. The structure of effector-free CRP has never been solved, although there is ample evidence that a significant conformational change occurs on cAMP binding (57, 79). The nature of the inactive form would be of significance for a variety of reasons, but primarily because it is impossible to understand the mechanism of protein activation in response to effector binding unless both the active and inactive states of the protein are known. Until recently, there were two lines of evidence that made specific predictions about the nature of the conformational change. The first was small-angle X-ray scattering analysis on CRP in the presence and absence of cAMP. Unfortunately, the results in the presence of cAMP were not interpretable because of aggregation, but the results in the absence of cAMP matched a model of a prolate elipsoid with an axial ratio of 1:2 (80). Because the crystal structure of the active form of CRP is roughly spheroidal, this implies a significant conformational extension of some portion of the protein without effector. The other frequently cited analysis involved determination of the Stokes radius by analytical gel chromatography (62). This method is somewhat indirect, however, since the results reflect not only the shape of the protein but also changes in solvent interaction, and these two factors are difficult to deconvolute. It is also a concern that the published analysis showed no change in global structure until cAMP levels exceeded 100 μM, well above those at which the protein should have bound two cAMP molecules. More recently, a nuclear magnetic resonance spectroscopy (NMR) analysis of inactive CRP suggested that the F helices are not solvent exposed, in contrast to the surface position of these DNA-interacting regions in the structure of active CRP (98, 100, 139). This is consistent with a very substantial conformational change on effector binding, although the exact nature of this change has been unclear (141).

A comparison of the structure of effector-bound CRP with that of effector-free CooA (Fig. 3) reveals differences that might be attributable to either the activation process or inherent differences in the proteins themselves. It is therefore difficult to draw conclusions about the former process. It is also unclear to what extent the activation processes in the two proteins are mechanistically similar. Nevertheless, it is worth considering the most central differences in the two structures: structure and positioning of the DNA-binding domains, structural differences within each effector-binding domain, and positioning of the two effector-binding domains with respect to each other.

Comparison of the DNA-binding domains of CRP and CooA shows that the individual domain structures are remarkably similar to each other (81), at least for regions that are resolved in each structure. However, it is the positioning of these domains with respect to each other and to the effector-binding domains that is very different in CooA and CRP. Irrespective of which of the two forms of inactive CooA is more similar to the solution form of the protein, they have important similarities: compared to the structure of active CRP, both forms are relatively elongated and have a dramatic repositioning of the DNA-binding domains, such that the F helices are actually turned away from the solvent. This latter point is consistent with the NMR analysis of inactive CRP noted above (141). These results lend credence to the hypothesis that CRP and CooA might undergo a roughly similar conformational change during activation. Effector binding must in some way signal a significant change in orientation of the DNA-binding domains in each protein. The signal transduction pathway that effects this change is of central importance to understanding these proteins.

Within the effector-binding domains, the obvious difference

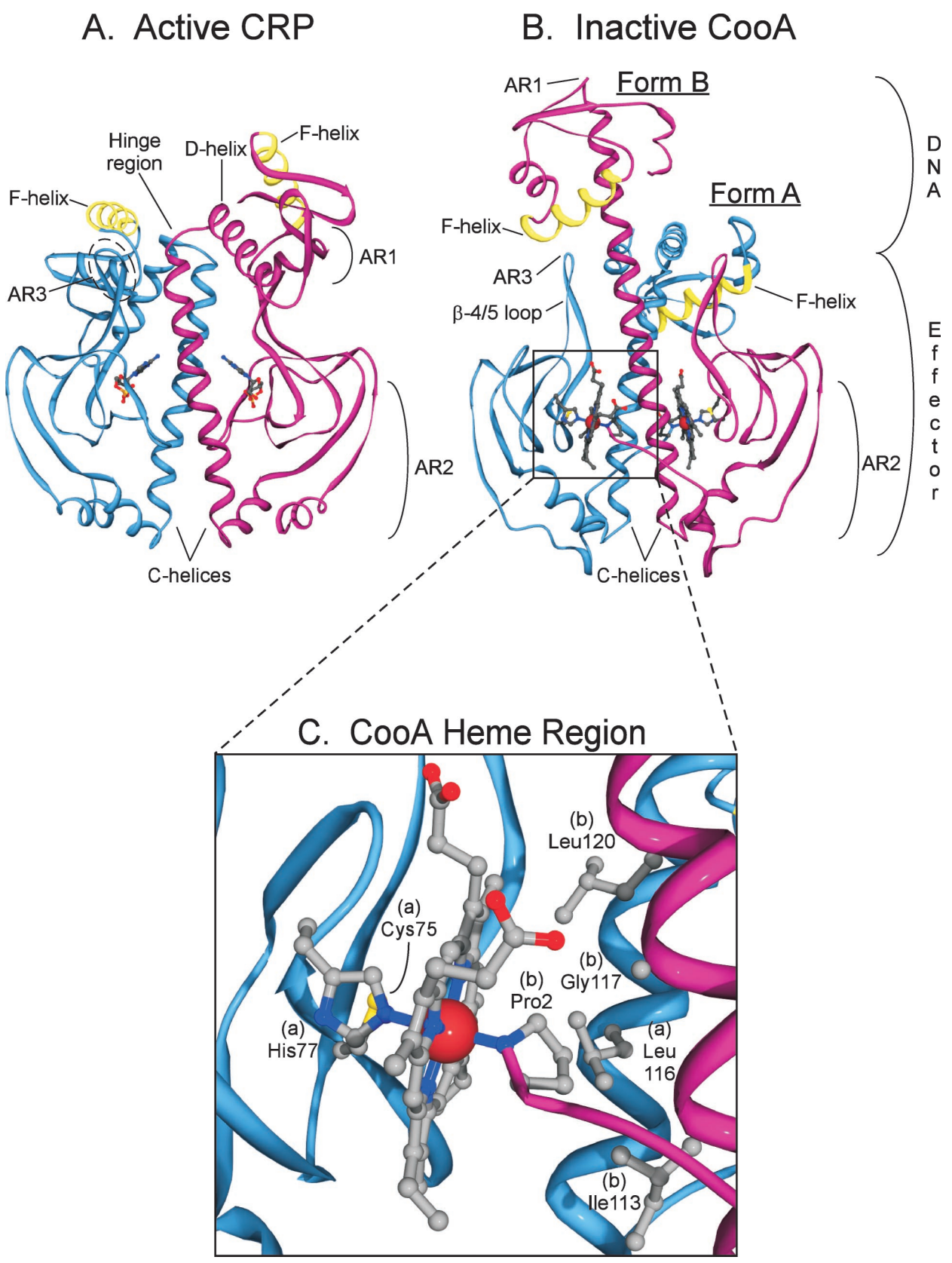

FIG. 3. Comparison of the structures of active CRP and inactive CooA. (A) Active CRP (Protein Data Bank no. 1G6N) is a symmetrical homodimer that is rotated slightly here to display critical features. The two monomers are colored differently, and the upper portion of each constitutes the DNA-binding domain, although this is difficult to see in this protein species. The F helices, which make specific DNA contact, are depicted in yellow, and other important helices are indicated. The approximate positions of the AR1 and AR2 regions are indicated on one

is the heme of CooA. The next most obvious difference between CRP and CooA is in the position of the 4/5 loop within each structure; the 4/5 loop refers to a pair of β strands that extend from the effector-binding domain in each protein toward the DNA-binding domain. While this change in position cannot be definitively attributed to the activation process, it is a very reasonable hypothesis, since the position of that loop in each protein predicts different contacts with the DNA-binding domains, presumably stabilizing each structure. Because the repositioning of the DNA-binding domains is certainly relevant to the activation process, it follows that surfaces within the effector-binding domain that contact the DNA-binding domain in either the active or inactive form of the proteins might also be repositioned after effector binding, a notion that is also addressed below where the protein surfaces that interact with RNA polymerase are discussed. It therefore seems highly likely that there are specific conformational changes within the effector-binding domains on effector binding. However, the actual nature of these structural changes remains poorly understood, except for those in the immediate vicinity of the cAMP in CRP and the heme vicinity in CooA.

The repositioning of the two effector-binding domains with respect to each other on effector binding is clearly central to the process of activation. This notion was first demonstrated by the Poulos group, who solved the effector-free CooA structure and compared it to that of effector-bound CRP (81). They noted a modest change in the relative position of the two long α helices, termed the C helices, that lie at the dimer interface of the two proteins. They suggested that this C-helix repositioning might serve as a signal transduction pathway between the heme region of CooA and the DNA-binding domains. As detailed below, this hypothesis has been strongly supported by direct mutational analysis of CooA. A similar notion was also proposed for CRP, where cAMP binding immediately adjacent to these helices might cause their repositioning (100).

In conclusion, a structural comparison of inactive CooA with active CRP shows that there is a substantial change in the position of the DNA-binding domains of CooA after CO binding and that repositioning about the C helices is a likely factor in that response. The data are also consistent with the notion that CRP and CooA undergo similar conformational changes on effector binding, but this speculation requires substantially more experimental testing. Understandably, the basis of CO recognition by CooA therefore requires an analysis of why CO leads to such a repositioning whereas other small molecules do not. This is addressed below.

Population Dynamics of the CooA Response to CO

To understand the response of CooA to CO, we must understand not only the structures of the predominant protein species in the presence and absence of CO but also the population distribution and dynamics among those different species. For example, the notion that CooA and CRP exist in completely inactive forms in the absence of effectors and exist in completely active forms in their presence is clearly simplistic. Rather, the active and inactive forms of the proteins must exist in a dynamic equilibrium, and the degree of homogeneity of either the active or inactive forms of either protein is unclear. For CooA, at least, it is our working hypothesis that its CO-bound form is inherently heterogeneous. The best evidence for this is that we have created CooA variants with substitutions based on the structure of active CRP that have severalfold-higher affinity for DNA in the presence of CO than does wild-type CooA under the same conditions. These substitutions do not lie near the DNA-contacting surfaces and are best explained by their creating a shift in the equilibrium such that a larger fraction of the CooA population is truly active. Another line of evidence discussed below, is from kinetic analysis, which has shown that there are two different states of the heme in a population of CO-bound wild-type CooA, as revealed by substantially different CO off-rates (103). Lastly, the failure to obtain crystals of the CO-bound CooA is consistent with the hypothesis of the heterogeneity of this form. Such a notion is easily rationalized on the basis of the physiological role of CooA. There is no detectable expression of the *coo* operons of *R. rubrum* in the absence of CO, suggesting that there is very little active CooA under these conditions. In the presence of CO, however, only two CooA-binding sites need to be saturated, and so it is reasonable to suppose that only a fraction of the CO-bound CooA need be in the form competent to bind DNA in order to achieve optimal gene expression. Because of the ease with which the structure of active CRP was solved, one assumes that CRP is homogeneous under these conditions. In any event, the impact on the presence of effector on the equilibrium of these two protein between their active and inactive forms is biologically important yet poorly understood.

Another complication involving a equilibrium between different protein forms probably exists with inactive CooA. In both forms of inactive CooA in the known X-ray structure, the F helices are buried from the solvent. However, one would expect that each of these regions would have some low affinity for a variety of DNA sequences. One might then predict that the very high concentration of DNA found in the cell might perturb the CooA structure by interaction with one or both of

monomer, and the AR3 region is circled on the other. cAMP is indicated by the pair of the ball-and-stick molecules near the center of each monomer. (B) The X-ray crystal structure of inactive CooA (Protein Data Bank no. 1FT9) shows that this protein exists in two forms, with the more extended form termed form B, although in each form the F helices are buried away from the solvent. DNA- and effector-binding domains are roughly indicated on the right side of the panel. Because of the extended structure, the AR3 region, at the tip of the β-4/5 loop, is easily seen. The heme region is depicted as the ball-and-stick structure and enlarged in panel C. (C) The heme vicinity of one CooA monomer is shown, with nearby residues noted and identified as to the protein monomer in which they are found: (a) refers to the monomer on the left, while (b) refers to the monomer on the right. His77 is shown as the ligand in the ferrous form, and Cys75; the ligand in the oxidized form, lies immediately behind it in this view. The other ligand, Pro2, is the N terminus of the right protein monomer and is connected to the red chain at the bottom right of this panel.

the F helices in a nonspecific way. If it is true that the actual structure of inactive CooA in the cell has a different arrangement of the DNA-binding domains from that depicted in the known structure, we will have difficulty in understanding which interactions stabilize and destabilize that structure. This would also be relevant to the analysis of the response of CooA to CO, since it would affect the actual nature of the CooA population that senses CO and therefore the pathway of CO activation.

Heme Vicinity: Structure and Implications

The residues in the vicinity of the heme are the features that provide the distinctive properties to different heme-containing proteins. For CooA, these residues are the basis for the specificity in CO binding and response. The heme vicinity of CooA (Fig. 3C) lacks the common heme-binding motifs found in the PAS-domain proteins or the globins (38, 122) but instead is somewhat similar to the effector domain of CRP. The critical residues governing this motif are highly conserved among CooA homologs, implying that there certainly are similarities among all of the CooA homologs in their heme-binding motif. The distal side of the CooA heme, where CO binds, consists of a pair of parallel α helices (the C helices), while the proximal side assumes a β-sheet structure. Once of the most important features of the CooA heme region is the presence of two endogenous protein ligands to the heme in inactive CooA. The incoming CO molecule must therefore replace one of the ligands in order to trigger the conformational change leading to activation of CooA (operationally, the incoming ligand binds to a five-coordinate heme that is created by the transient deligation of an endogenous ligand). This requirement in CooA for displacement of an endogenous ligand is unusual among heme proteins and helps explain the specificity of CooA for CO. Most small molecules are not sufficiently strong heme ligands to displace these endogenous ligands, while NO displaces both protein ligands and creates a five-coordinate heme. This NO adduct is not active, consistent with the notion (explained below) that tethering of the heme by the endogenous His77 ligand is critical for CooA activation. Oxygen oxidizes the heme, and that form of CooA is also inactive. Part of the specificity of CooA for CO therefore relies on the fact that only CO has the appropriate ligand strength to displace one, but not both, of the endogenous heme ligands.

His77 serves as one heme ligand in reduced CooA and is critical for the response of CooA to CO, because substitution with any other residue at that position destroys the CO-dependent response of the protein (116; M. Conrad, H. Youn, and G. P. Roberts, unpublished data). His77 is important for two reasons. First, the His ligation is at a critical poise of ligand strength. It must be sufficiently strong to avoid displacement by CO or other small molecule ligands, since such binding to the "wrong side" of the heme does not allow proper C-helix repositioning. However, it must also not be so strong that a six-coordinate NO adduct is formed, since such a species might well be active. The second important property of His77 is that its serves as the tether to the CO-bound heme. Its precise size and positioning are therefore important for the precise positioning of the CO-bound heme with respect to the C helices, and this last interaction is important for activation of CooA. The ligand strength and positioning of His77 must also be relevant to the proper redox-mediated ligand switch between Cys75 and His77 that is described immediately below. It is therefore not surprising that all CooA homologs have a His residue at the homologous position of His77 (Fig. 4).

On the His77 (proximal) side of the heme are two other residues of importance to CO sensing. The first is Cys75, a heme ligand in Fe(III) CooA that is displaced by His77 on reduction (6, 107, 116). While Cys75 is not a ligand in the active form of CooA, mutational studies of *R. rubrum* CooA have nevertheless shown that only Cys and Ser allow high CooA activity in vivo, while Ala allows some CooA function. The similar size of these residues suggest that larger residues might either perturb heme insertion and stabilization or interfere with proper positioning of the heme on CO binding. The sequences of the CooA homologs are consistent with this, since only Ser and Cys are found at that position. In *R. rubrum* CooA, the role of Cys75 as the ligand in the oxidized form predicts that its presence might be critical for stabilization of that form and that Ser, which cannot serve as a heme ligand, would not suffice. A C75S variant of CooA does show some heme stability problems when oxidized, but, surprisingly, there is a substantial population of six-coordinate heme in the oxidized form of this variant, implying that another protein residue is serving as an adventitious ligand (116). The identity of this residue is unknown. The role of Cys75 in oxidation-reduction is addressed in the following section.

Asn42 is the other proximal-side residue (in addition to His77) that is directly perturbed when CO binds to the heme. This residue makes H-bonding contacts with His77 in the reduced form but not in the CO-bound form (24, 81). Since His77 is tethered to the heme in both forms, this structural change of His77 suggests a repositioning of His77 with respect to Asn42 in response to CO. CooA variants with substitutions at this position are somewhat perturbed in their ability to be activated by CO, but the precise basis for this is not clear (24). It is also interesting that the adjacent residue is Glu41, which has an effect on CooA-RNA polymerase interactions (82). This suggests that CO binding might have a direct effect on this interaction as well, as discussed below.

The other ligand in the reduced form of CooA, Pro2, is the N terminus of the other protein monomer (81). Proline had not previously been detected as a heme ligand in any protein because it is sterically incapable of playing that role except when it happens to be at the N terminus. The presence of such a novel ligand immediately suggested that it might be critical for the proper activation of CooA, but mutational analysis has disproved that (125) and has shown that a variety of substitutions at this position provide substantial CooA activity. This view is supported by the observation that none of the other CooA homologs appears to have a proline positioned to serve as a ligand (Fig. 4). However, while Pro2 is not critical for the function of CooA in *R. rubrum*, it does appear to be optimal.

Another residue is important in stabilizing Pro2 as a ligand, and that is Arg4, which appears to interact with a propionate of the heme (81). Removal of this residue by deletion results in a detectable population of five-coordinate heme in both the oxidized and reduced forms of CooA (125).

When CO binds to the heme of CooA, it replaces one of the two protein ligands, but the identity of the displaced ligand was unknown for some time. In part this reflected the fact that

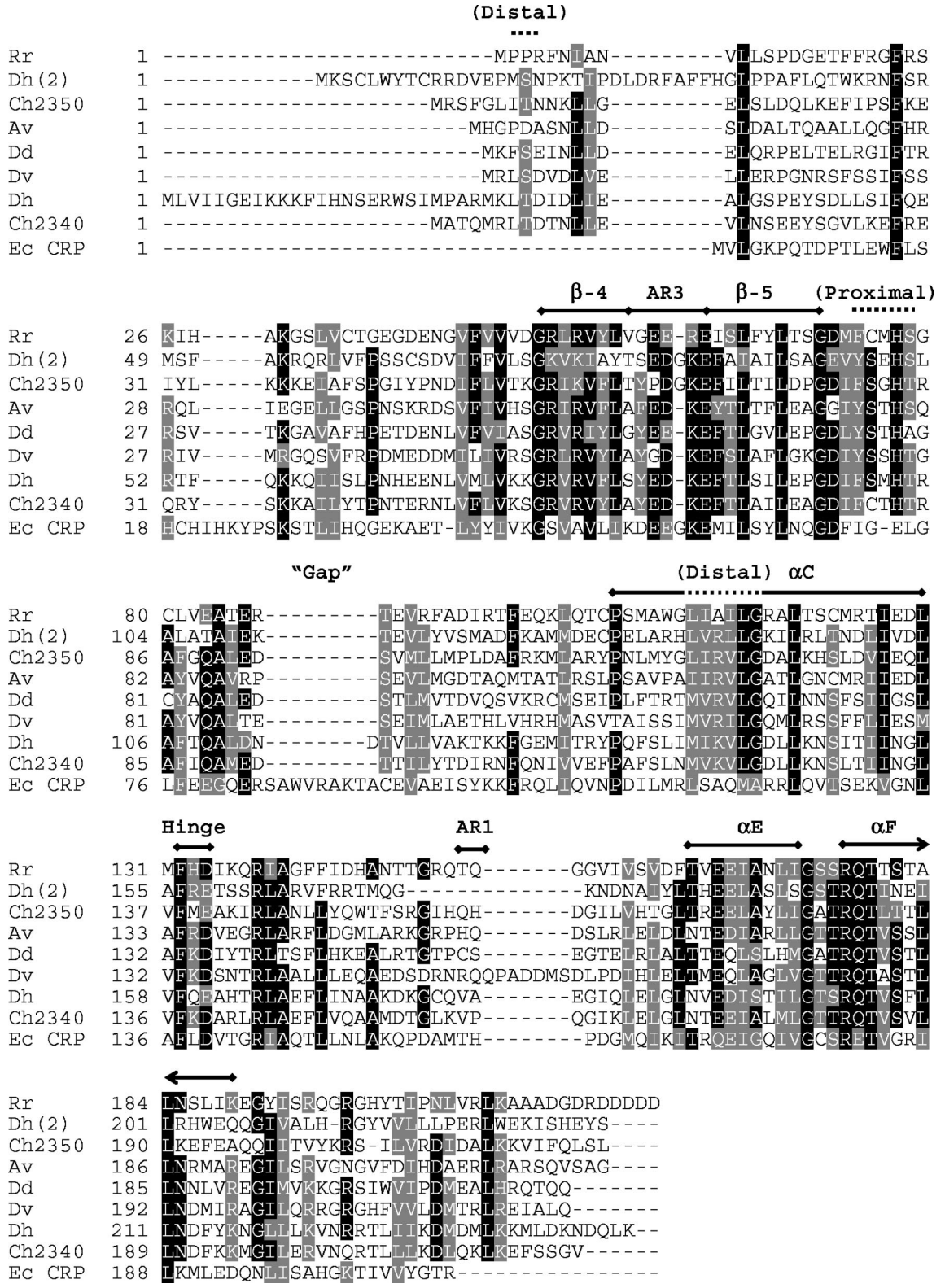

FIG. 4. Sequence alignment of the CooA homologs (reprinted from reference 147). This shows the sequence comparison of the CooA homologs described in the text, with CooA of *R. rubrum* shown at the top and, for comparison, the CRP of *E. coli* at the bottom. Residues that are extremely or modestly conserved among the CooA homologs are shaded in black and gray, respectively. Above the top line, specific α-helix or β-sheet regions are noted, as are the following important regions: the distal heme pocket, which is formed both by the N-terminal residues and those around positions 112 to 117 in *R. rubrum* CooA (dotted lines); the proximal heme pocket, including Cys75 and His77 (dotted lines); the β-4 and β-5 regions forming the 4/5 loop and flanking the AR3 region; the portion of CooA deleted relative to CRP (termed "Gap"), which provides space for the heme; the hinge region with Phe132, which separates the effector- and DNA-binding domains; AR1, by analogy to a critical residue in CRP; and the E and F α helices, which define the DNA-binding region, with Gln178 shown near the beginning of the F helix. Rr, *R. rubrum*; Dh, *D. hafniense*; Ch, *C. hydrogenoformans*; Av, *A. vinelandii*; Dd, *D. desulfuricans*; Dv, *D. vulgaris*; Ec, *E. coli*.

there was no spectroscopic data set for the novel Pro ligand to serve as a control for the CO-bound form of CooA. The issue was resolved by the application of NMR by the Aono group, which showed that CO replaces Pro2 (144). A resonance Raman analysis has indicated that the displaced Pro2 is not in the immediate vicinity of the bound CO (24). This result is consistent with the observation that alteration of Pro2 in CooA does not dramatically impair the ability of the protein to achieve the active conformation (125). However, there appear to be three auxiliary roles for Pro2 in *R. rubrum* CooA function. The first is that its ligation to the heme helps keep the protein in the inactive form until CO binds. In an otherwise wild-type background, alteration of Pro2 does not yield a substantial increase in CO-independent activity, which would be the expected result if Pro2 ligation were critical for this role (125). However, an involvement of Pro2 in this process is revealed in backgrounds with other substitutions that enhance CO-independent activity and in which the replacement of Pro2 is synergistic for this response (71). We assume that the modest effect seen in an otherwise wild-type background is because of the presence of other unidentified protein ligands that can adequately maintain the inactive form. The second role of Pro2 is that it provides a heme ligation that is weak enough to be displaced by CO yet strong enough to resist displacement by weaker small-molecule ligands. It is not clear if the residues that replace Pro2 in variants of *R. rubrum* CooA or in the CooA homologs have similar properties, because binding of other small molecule has not been examined with these proteins. Finally, Pro2 and its adjacent N-terminal residues must be flexible enough to remain ligated to the heme through the oxidation-reduction process. As explained in the following section, this process involves a significant movement of the heme relative to the protein, requiring ligand flexibility.

In the CO-bound form of CooA, the residues presumed to be near the heme-bound CO are all from the two C helices at the dimer interface. These include Leu112, Ile113, Leu116, Gly117, and Leu120 (146). The evidence for the rolles of these residues is presented below, but some general comments are appropriate here. It is important to recognize that the structure of the CO-bound form of CooA is unknown, and so the exact position of the CO-bound heme with respect to amino acid residues is not clear. Despite these uncertainties, a number of CooA variants altered at positions 113, 116, and 117 show perturbations in the C—O and Fe—C stretching frequencies as determined by resonance Raman spectroscopy (24). This suggests that the bound CO is located near these residues. As detailed below, some of these residues are critical for the activation of CooA in response to CO. Other substitutions in this region create CooA variants that respond effectively to imidazole as an effector (146; H. Youn, R. L. Kerby, and G. P. Roberts, unpublished data), consistent with a role of this region in interacting with the small molecule bound to the heme. In the CooA homologs, Leu116, Gly117, and Leu120 are all strictly conserved while positions 112 and 113 have conservative substitutions (Fig. 4). These results are consistent with the hypothesis that it is the interaction of this portion of the protein with the CO-bound heme that leads to the repositioning of the C helices in the normal activation process.

In summary, there are two obvious local changes in the vicinity of the CooA heme in response to CO: displacement of proximal Pro2 ligand, which allows repositioning of the CO-bound heme with respect to the C-helix residues, and breakage of the His77-Asn42 H-bond. This combination of features in the unique CooA heme-binding motif ensures that only CO can trigger the structural rearrangement necessary for activation.

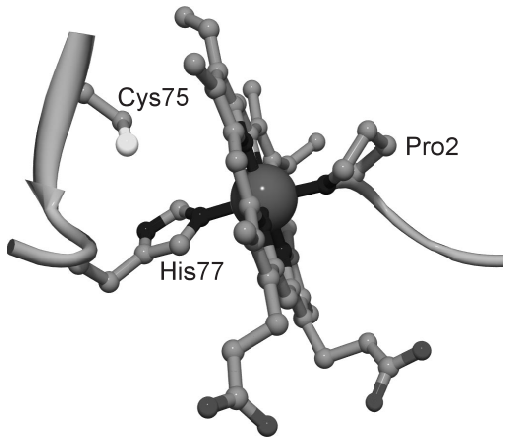

FIG. 5. Structure of the heme in the reduced form of CooA, oriented to show the relative positions of Cys75, which serves as the ligand in the oxidized form, and His77, which serves as the ligand in the reduced form. The Cys75 and heme Fe must move 2 to 3 Å with respect to each other for this ligation to occur.

The Oxidation-Reduction Mechanism in CooA and Its Implications

It makes sense that the facultative aerobe *R. rubrum* would avoid expressing the *coo* operons under aerobic conditions, because the CODH is itself oxygen labile. However, the situation is actually more interesting than that. The NiFe metal center of the CODH is catalytically active only at reduction potentials below −300 mV (60), which also has been reported to be the midpoint potential of the transition of CooA from the oxidized to the reduced form (94). CooA of *R. rubrum* solves the physiological problem of avoiding *coo* expression under oxidizing conditions in the following way. The heme of CooA can bind CO only when reduced, and so the oxidation of the heme, either by O_2 or by other oxidants, prevents CooA activation even in the presence of CO. Mutational and spectroscopic analyses have shown that there is a highly unusual ligand switch after the oxidation and reduction of CooA: oxidized CooA has Cys75 as ligand, but this is displaced by His77 on reduction (6, 116). It seems likely that this ligand switch sets the specific poise for heme reduction, but this assumption has not been tested experimentally. The structure of the oxidized form of CooA remains unknown, but examination of the structure of reduced CooA makes it clear that this ligand switch involves a significant movement of the heme with respect to the protein backbone (111) (Fig. 5). Indeed, this observation was one of the facts that made it clear that there was substantial flexibility in the heme position, a notion that has been expanded in our current hypothesis of heme repositioning as an essential feature of CO activation.

Rather interestingly, all the CooA homologs have Ser at the

position homologous to Cys75 of *R. rubrum* CooA position except *C. hydrogenoformans* 2340 CooA, which also has Cys. The particular CooA homologs that have been partially purified and studied in vitro all have the ability to be oxidized. However, even in *C. hydrogenoformans* 2340 CooA, it appears that the ligation structures of oxidized and reduced forms, as well as the redox poise of the transition, are somewhat different from those seen with *R. rubrum* CooA (147). This results implies that while Cys75 is important for the precise nature of the ligand switch seen in *R. rubrum* CooA, it is not sufficient, and that other residues in the heme vicinity are important for this property.

A number of interesting and biologically significant questions concerning the redox switch in CooA remain unanswered. One is obviously the exact nature of the conformational change that occurs within the effector-binding domain to not only allow this switch but also stabilize both forms of the protein. At present, we have relatively little insight into the homogeneity of either of these species. Indeed, the original analysis of CooA redox properties showed a curious hysteresis such that the curves obtained for oxidation were distinct from those obtained for reduction. This behavior was rationalized by a very slow interconversion between the two forms (94), yet a different analysis by the same group revealed that the conversion occurred in the millisecond range (93). The basis for this discrepancy is unknown. A second question concerns the identity of the ligand *trans* to Cys75 in oxidized *R. rubrum* CooA. Indirect evidence also suggests that Pro2 serves as the ligand *trans* to Cys75 in the oxidized form of CooA (125, 145). Finally, a more biologically interesting question concerns the actual chemical entities sensed by CooA for this redox transitional, though O_2 can certainly suffice. Presumably it is some pool of small molecules such as NAD and NADH, but the identity of that small molecule remains unknown.

Basis of the CO Specificity of *R. rubrum* CooA

There appear to be two distinct aspects of the remarkable CO specificity of CooA: only CO can displace the appropriate heme ligand to form a six-coordinate form, and only CO, when bound to the heme, can stimulate the proper conformational change to activate the protein. The following discussion explains the basis for each of these properties and their role in CooA activation.

We have already explained that the strength of the endogenous protein heme ligands can explain the remarkable CO specificity of *R. rubrum* CooA for its activation. The simple hypothesis was that only CO could form a six-coordinate species by displacing the Pro2 ligand and that this form might therefore be both necessary and sufficient for activation. The obvious prediction was that perturbation of the Pro2 ligation could weaken that bond and allow other small molecules to bind the heme on the proper side. This happens to be true, based on the following analysis of the ΔP3R4 variant of CooA, in which the codons for the third and fourth residues have been deleted. This alteration eliminates the Arg4 residue that stabilizes Pro2 ligation to the heme, producing a small but significant population of five-coordinate heme in the reduced form. Not surprisingly, this variant is able to bind CN^- and imidazole very efficiently, but binding of these molecules does not activate the protein to a detectable extent (146). This result disproves the simple hypothesis above and indicates that there is another level of discrimination for CO. What might be the basis for this discrimination, especially against CN^-, which is so similar to CO in size?

It is clear that the bound CO exists in a very confined pocket in *R. rubrum* CooA, because rebinding of CO after its removal by photolysis is unusually rapid and efficient (5, 112, 130). Because the structure of the active form has not been solved, the identity of this pocket is unknown, but it is apparently not formed by the N terminus, as evidenced by the resonance Raman results cited above. It is therefore presumed that the pocket must be formed by the only other residues in the heme vicinity, which are those on the C helices of both protein monomers. The nature of this pocket is of interest for two biological reasons that are explained further below. First, the interaction of the CO-bound heme with the C helices is almost certainly a critical step in signal transduction within CooA since it causes the C-helix repositioning necessary for activation. Second, as described immediately below, the nature of the interactions in this heme pocket must certainly play an important role in the specificity of the CO response.

Under the hypothesis that this CO specificity results from a precise interaction between the CO-bound heme and the C-helix residues, a number of these have been analyzed by randomizing the codons singly or in small groups and then screening for variants that responded to CO. The expectation was that certain positions should be critical for a response to CO. The presumption that these residues were in the general vicinity of the bound CO was supported by the observation that certain substitutions at positions 113, 116, and 117 (Fig. 3C) perturbed the CO stretching frequency in resonance Raman analysis (24). In fact, only Gly117 was absolutely required for a CO response, although position 120 was also fairly stringent, tolerating only Ile in place of Leu120 (146, 147; R. L. Kerby and G. P. Roberts, unpublished data). While it is tempting to suppose that these might be the residues that determine CO specificity, this hypothesis has been weakened by the results presented below for CooA variants that respond to imidazole.

In a similar analysis, a variety of hydrophobic residues were found to be acceptable at positions 112, 113, and 116. However, the analysis provided the important observation that hydrophilic residues at these positions cause a decrease in the accumulation of heme-containing CooA and were also unable to respond to CO (24, 146). The first effect is consistent with the idea that a hydrophobic pocket is typically found around hemes and presumably serves to maintain the heme in the protein. The second result suggested the following hypothesis to explain why CO binding to the heme might lead to C-helix repositioning. CO binding to the heme displaces the Pro2 and its attached N terminus, which apparently moves away from the heme. This then exposes the largely hydrophobic surfaces of the C helices to an aqueous environment. The repositioning of the C helices that results in activation might then be the result of an effort to reduce the solvent exposure. Alternatively, hydrophilic residues at these positions might interfere directly with the proper C-helix positioning or indirectly by affecting heme positioning. The result of the above analysis was to suggest that Gly117 and Leu120 might make critical contacts with the bound CO, where the other residues were less likely to

do that because a variety of hydrophobic residues at those positions allowed a fairly normal response to CO (146).

Concurrent with the analysis of the C-helix requirements for a proper response to CO, we analyzed the same region of CooA for its ability to allow activation by imidazole. Recall that the ΔP3R4 CooA variant is able to bind CN^- and imidazole but is not activated by them. Under the assumption that the additional level of ligand specificity probably was due to residues in the vicinity of the bound ligand, we therefore started with ΔP3R4 CooA, randomized various C-helix residues, and screened for activation in response to imidazole in vivo. Randomization of positions 117 and 120 yielded no imidazole-responsive variants, but the simultaneous randomization of position 113 and 116 did (Youn et al., unpublished). A variety of combinations of residues at these positions supported this phenotype, but the striking commonality was the presence of a Trp residue at one of the two positions. The basis for this is unknown, and it is clear that other aromatic residues are much less effective. The majority of the imidazole-responsive variants continued to be activated by CO as well, but some, such as ΔP3R4 Trp113 Trp116 and ΔP3R4 Arg113 Trp116, were substantially more active in response to imidazole than in response to CO. This result shows that these positions are critical for the imidazole response, presumably by some interaction with the bound imidazole itself, although more complicated mechanisms cannot be ruled out.

One imidazole-responsive CooA variant (ΔP3R4 Trp113 Trp116 CooA) was then further analyzed for the importance of Gly117 and Leu120. The rationale was that if either of these residues provided a precise contact with the bound CO or, in a related way, served as the basis for CO specificity, then the requirements at these positions would be very different for a response to imidazole. In each case, only the wild-type amino acid residues were acceptable at these positions. While this does not disprove the notion that these residues make specific contacts with the heme-bound CO in wild-type CooA, it is much simpler to imagine that there are similarities in imidazole and CO responsiveness and that these residues are both critical in that shared pathway. The nature of the shared pathway would probably be the C-helix repositioning described below.

While it is therefore obvious that there is another level of CO specificity in CooA, the molecular basis for it remains unclear. Our current hypothesis is that the CO-bound heme must move to a hydrophobic region along the C helices and that this movement is precluded by ligands other than CO. Imidazole is both bulky and hydrophilic, so that its movement into such a pocket is prevented, while the charge on CN^- would also prevent its presence in a hydrophobic pocket. However, if imidazole is too bulky for normal activation, what is the basis of the imidazole-responsive variants that have been detected? Obviously the exact nature of the active forms of these variants is unclear, but our working hypothesis is that their precise mechanism of activation is different from that of wild-type CooA in response to CO. In other words, we imagine that the imidazole-bound heme interacts in a different way with the modified C-helix residues from the way in which CO interacts with the normal residues but that these different interactions both have the common result of C-helix repositioning. We then imagine that residues 117 and 120 are involved in that shared pathway. This result with the imidazole responders is particularly interesting since it indicates that CooA and its variants sense CO and imidazole by mechanistically different processes. In contrast, the models explaining the response of FixL to different small molecules assume that the sensing system of the protein is essentially identical for each effector (56).

A recent observation is also consistent with heme movement. Kinetic analysis has revealed that CO-bound wild-type CooA is heterogeneous in terms of the CO off-rate (103). One population shows a very low off-rate, consistent with the tight CO pocket already reported (112, 130). However, a roughly comparable population displays a significantly higher off-rate, implying a different position of the CO-bound heme. These two populations are in slow equilibrium, suggesting that a substantial conformational change might be occurring in the transition. This result is consistent with the notion that the two populations detected by this method might reflect the populations of active and inactive CO-bound CooA described above.

While we do not know the precise position of the CO-bound heme in CooA, it remains a tantalizing possibility that on CO binding, the heme approaches the position occupied by cAMP in active CRP. If this is correct, then the two proteins might be responding to their respective effectors in fundamentally similar ways. Determination of the mechanistic similarities and differences between the two proteins continues to be a focus of research because it should reveal commonalities for other members of the family of related proteins as well.

Repositioning of C Helices as a Signal Transduction Mechanism

The initial observation that suggested that repositioning of the C helices of CooA might be an important signaling pathway between the heme region and the DNA-binding regions was the comparison of the structures of active CRP and inactive CooA (81). However, because a comparison of such different proteins is obviously problematic, a more direct test was performed as follows.

CooA, together with its homologs and also CRP and FNR, has a leucine zipper motif in the paired C helices. However, an analogous heptad repeat in the leucine zipper of all of these proteins, which lies about one-third of the way down the helices from the hinge region (positions 121 to 126 of CooA), is poor in comparison to a leucine zipper consensus. This led to the hypothesis that this nonconsensus heptad permitted flexibility in the structure, allowing a transition between an active and inactive form. Support for this notion for CRP has been made on structural grounds (100), and it is interesting that the D154A substitution that allows FNR to be active under aerobic conditions also affects this region (76). We reasoned that if helix repositioning was the signal pathway for CooA, then creating such a repositioning by mutation should short-circuit the signal and provide effector-independent activity. We therefore randomized the codons for positions 121 to 126 in an otherwise wild-type CooA background and screened for CO-independent variants (71). Sixty variants were sequenced, displaying a variety of different phenotypes, but all variants with substantial CO-independent activity had Leu residues (or other appropriate residues for a leucine zipper) at positions 123, 124, and 126. This is a fairly clear result and identifies

helix repositioning as a major signal pathway within CooA. As noted above, the notion has also been proposed for CRP (100).

CooA variants with improved leucine zippers have substantial activity without CO but also show a further increase in activity, to approximately the wild-type level, in the presence of CO (71). Apparently that repositioning of the C helices is only partially effective at shifting the equilibrium to the active form if CO was not bound to the heme. There are two general possibilities to explain this. First, in the absence of CO, the improved leucine zipper variants might be under competing forces, with the continued Pro2 ligation to the heme preventing a full and proper repositioning. Second, CO binding to the heme might cause other conformational changes within the effector-binding domain that also assist in activation of the protein. In other words, while the C-helix repositioning is very important, it might not be the only signal pathway. In fact, both of these possibilities appear to be true.

The apparent tension caused by the retained Pro2 ligation was shown as follows. Among the CooA variants randomized at positions 121 to 126, one of the most active without CO had Ala121 and Gly122 substitutions. We noted that these substitutions lie between the improved leucine zipper region and the region of the C helices that are near the heme. These substitutions might therefore create a bend or a flexible region in the C helix, reducing the adverse tension in the absence of CO. Consistent with this, when the same pair of residues were introduced into an otherwise wild-type CooA background, the response to CO was diminished. This is reasonable because it is the rigidity of the helices in wild-type CooA that should be necessary for signal transduction through the protein. Subsequent analysis of that vicinity of the C helices is consistent with this idea, although there are contacts with other parts of the protein that complicate the analysis (71). A different confirmation of this model involved the addition of the ΔP3R4 substitution to the improved leucine zipper background. By itself, the ΔP3R4 causes negligible CO-independent activity in vivo, presumably in part because an adventitious ligand is able to satisfactorily replace Pro2 in keeping CooA inactive without CO. However, in the improved leucine zipper background, ΔP3R4 allows very high CO-independent in vivo activity. This is easily rationalized by the fact that this variant can no longer efficiently tether Pro2 to the heme (nor can the adventitious ligand do this with the same effectiveness as Pro2) and therefore cannot effectively interfere with the C-helix repositioning caused by the improved leucine zipper.

The second possibility, that CO binding sends activation signals by other mechanisms, also has some support. As described below, there is good evidence that CO binding directly alters the positioning of some of the regions of CooA that interact with RNA polymerase. It is less clear that there is another pathway between the CO-bound heme and the DNA-binding domains, but this is certainly a reasonable possibility based on the structure. A comparison of the structures of active CRP with inactive CooA indicates a very different positioning of the 4/5 loop in each protein, and there are clear contacts between this loop and the DNA-binding domain of active CRP. Finally, because His77 is directly connected to the 4/5 loop that starts at approximately residue 69, any movement of the heme after CO binding would be expected to move the tethered His77 and its attached protein backbone as well. However, there is no experimental evidence in support of such a pathway in CooA.

The model of C-helix repositioning is also supported by data from a completely different set of CooA variants. These variants have been found in different mutageneses and screens for effector-independent variants under various conditions. One of the most compelling cases is that of L116K CooA, which is active when reduced but actually loses activity in the presence of CO (149). A variety of spectroscopic analyses have suggested, albeit indirectly, that this variant is altered in its ligation state and that Lys116 appears to replace Pro2 (the position of Pro2 is shown in Fig. 3C). Modeling such a Lys116 ligation on the known structure of reduced CooA requires a substantial movement of the heme with respect to the C helix. The activity in the reduced form of L116K CooA is therefore probably the result of helix repositioning by a direct covalent bond between the heme and the C helix. This is in contrast to the mechanism already proposed for wild-type CooA, where helix repositioning results from the exposure of the hydrophobic pocket or from heme movement along the hydrophobic C helices.

Whereas important details concerning the exact interaction between the CO-bound heme and the C helices remain to be discovered, the above results provide fairly conclusive evidence that this is a major mechanism for transmission of the of the CO-binding signal through the protein to the DNA-binding regions.

Achieving the Active Structure of CooA

Nature of the active form of CooA. Our image of the active form of CooA is based largely on the crystal structure and related data for CRP. Because of that and because the information is not central to the response of CooA to CO, we will merely summarize the information here.

(i) The heme in active CooA is certainly deliganded from Pro2 and appears to move with respect to the protein portion of the effector-binding domain, although it certainly remains tethered to His77. While we have hypothesized that the heme might move along the C helices, this has not been demonstrated.

(ii) In the active form of CooA, the C helices have undergone a small but important reorientation relative to each other.

(iii) The DNA-binding domains are arranged in a fashion likely to be that of active CRP, because the sequence bound are very similar. This is discussed in more detail in the following section.

(iv) The hinge region between the DNA- and effector-binding domains (roughly Phe132 in CooA and Phe136 in CRP) is dramatically rearranged. In CRP, Phe136 makes contact with the 4/5 loop, which might be an important interaction for stabilizing the active form. While this notion has not been tested experimentally for CRP, Phe is the only residue at that position that provides significant CO-dependent activity to CooA (H. Youn and G.P. Roberts, unpublished data). This result is consistent with the absolute conservation of a Phe at this position among the CooA homologs.

(v) The 4/5 loop is in a position to interact with the DNA-binding domain, the hinge region, and other residues on the C helix. This centrality to a number of regions of the protein that are likely to be critical to the stability of the active form sug-

gests that the apparent movement of the 4/5 loop from the position in inactive CooA to the position in active CRP is another major aspect of the activation mechanism.

Interaction of CooA with specific DNA sequences. CooA is known to bind to two naturally occurring DNA sequence in the *R. rubrum* genome, which are reminiscent of the sequences bound by CRP and FNR (42, 58, 79). Because a mutational analysis of the binding sites of CooA has not been performed and because of the paucity of naturally occurring sites, it is premature to speak about a consensus half-site. Nevertheless, the half-sites are so similar to each other that some general comments can be made. For both CRP and CooA, these regions have two 5-base inverted repeats, termed half-sites, separated by six other bases. The striking difference in these sequences is the C at the fourth position of the half-site bound by CooA (TGTCA), where a G is used by CRP(TGTGA). This can be rationalized by examination of the F helices of CRP and CooA, since the Glu181 of CRP, known to make direct contact with the G base, is replaced by a Gln (Gln178) in CooA of *R. rubrum* and in all of its homologs, although the specificity of this residue for C base in this context has not been tested experimentally. The T in the middle of the palindromes for all three proteins does not seem to be contacted by any residues in the CRP structure, and it is a reasonable hypothesis that it is necessary for the 30° bend that is known to be induced in the DNA by CRP on binding (79). FNR uses a generally similar F helix, though its exact DNA target sequence is slightly different (9).

For CRP, a consensus sequence was guessed from the comparison of many naturally occurring CRP binding sites, and this consensus displayed a dramatically higher affinity for CRP in vitro than does any natural site (54). This implies that the physiologically appropriate affinity of CRP for DNA must be lower than the maximal possible and that each individual CRP-binding site achieves that affinity by different perturbations from the consensus sequence. As a consequence, most naturally occurring CRP-binding sites are discernibly different in their sequences. With this background, it is therefore a bit surprising that the four CooA half-sites have only a single example of a substitution. It seems unlikely that all half-sites would coincidentally diverge from the highest-affinity sequence in the same way. It is therefore possible that CooA achieves the biologically appropriate affinity with an F helix for which there is no DNA sequence with the very high affinity produced for CRP by its consensus sequence. However, a more careful comparison of the affinity of CRP and CooA for their respective DNA sequences and an analysis of the affinity of CooA for other sequences should clarify this situation.

Positioning CooA for proper interaction with RNA polymerase. It is obviously central to their biological role that CooA, CRP, FNR, and related proteins form proper interactions with RNA polymerase in order to stimulate transcription. While a great simplification, their regulated promoters have relatively poor affinity for RNA polymerase by themselves and the presence of one of these activator proteins bound near the promoter provides protein-protein contacts that make polymerase binding more energetically favorable. CRP and FNR appear to bind at two types of sites: in the enteric bacteria, class I sites are centered from −61.5 to −93 relative to the start site of transcription, while class II sites are typically centered at −41.5

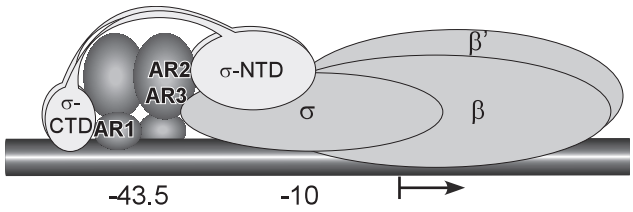

FIG. 6. The positioning of CooA with respect to RNA polymerase indicates that the two CooA monomers have fundamentally different interactions at class II promoters. The upstream monomer makes contact with the carboxyl-terminal domain of α (α-CTD), at the AR1 region, while the downstream monomer contacts the σ subunit at AR3 and the amino-terminal domain of α (α-NTD) at AR2.

(8, 16). This difference in positioning with respect to the promoter implies rather different contacts between the activator and the polymerase, but we discuss only the class II promoters, since that is the class into which the two natural promoters for *R. rubrum* CooA fall (42, 58). The work with *R. rubrum* CooA in an *E. coli* background has also used a binding site/promoter of this class.

At class II promoters, at least three different regions on the activator come into contact with RNA polymerase, and these regions are termed AR1, AR2, and AR3 (for "activating regions") (15) (Fig. 6). Because of the geometry of the activator dimer and RNA polymerase, each monomer makes specific interactions as follows. AR1 exists only in the upstream monomer, relative to RNA polymerase, and is found primarily in the DNA-binding domain of that monomer. It makes specific contacts with the carboxyl-terminal domain of the α subunit of polymerase, which reaches over the activator protein on a long, flexible arm (8, 95, 96). AR2 lies in the effector-binding domain of the downstream activator monomer and makes specific contacts with the amino-terminal domain of the α subunit (95, 96). Finally, AR3 is also in the effector-binding domain, specifically the 4/5 loop of the downstream monomer, and makes contacts with the σ subunit of polymerase (8, 84). The relative importance of each specific AR is different in CRP and FNR (109, 140), but there is no reason to suspect that the geometry of the interacting complexes is profoundly different.

CooA has all three ARs, at least when activating transcription with the heterologous RNA polymerase from *E. coli*. Both gain-of-function and loss-of-function variants affecting AR2 and AR3 have been described (82), and the presence of an AR1 was revealed by the requirement for a functional carboxyl terminus of the α subunit for in vitro transcription (59).

The proper positioning of the ARs for interaction with RNA polymerase is a direct result of the proper orientation of the DNA-binding domains. This is clear, for example, because AR1 is in the DNA-binding domain and the positioning of the F helix of the latter for DNA interaction must also position the AR1 for RNA polymerase interaction. Similarly, because AR3 is at the tip of the 4/5 loop and it seems likely that this loop is positioned in the active form through interaction with the DNA-binding domain, it seems likely that this region also should necessarily be properly positioned. It is less clear whether there is repositioning of other AR surfaces, predominantly AR2, on effector binding that is independent of the DNA-binding domain movement. In other words, is AR2 nec-

essarily in the proper position for the RNA polymerase interaction when the DNA-binding domains are positioned to bind DNA, or must it be separately positioned in response to effector binding?

There is suggestive evidence in support of this latter idea for both CooA and FNR. In CooA, Glu41 is important for interaction with the RNA polymerase (82) and is adjacent to Asn42, which makes direct contact with a heme ligand, His77 (81). It is therefore a reasonable possibility that CO binding to the heme might affect the precise positioning of Glu41 through the repositioning of the heme and therefore of Asn42 (81). Specific changes at a few other CooA residues in the heme vicinity also have the effect of perturbing activation without perturbing DNA binding. These include Met76, Phe74, and possibly others (M. Conrad, R. L. Kerby, and G. P. Roberts, unpublished data). However, to date, the data are merely consistent with the hypothesis of a direct effect of CO on AR positioning, and it has not been rigorously demonstrated. A similar notion has been proposed for FNR as well, where a C20S substitution that alters a ligand to the 4Fe-4S cluster is defective in transcription activation yet appears to bind DNA effectively based on its ability to bind as a repressor (114).

Although the hypothesis of a separate and direct positioning of certain AR surfaces in response to effector binding seems initially surprising, it probably should not be. It is clear for all of these activator proteins that effector binding causes a substantial conformational change within the effector-binding domain, in addition to a repositioning of the DNA-binding domains. Given that the AR2 surfaces are adjacent to the regions of the protein that bind the effector, it is actually a reasonable prediction that the hypothesis should be true for at least some AR residues. Presumably the positioning of the AR in the DNA-bound form of the protein would be more appropriate for interaction with RNA polymerase than would that of the effector-free form.

Would there be a biological consequence of direct AR positioning by effector binding? Based on the equilibrium model proposed above, it seems that there will always be a subpopulation of activator protein that positions the DNA-binding domains properly in the absence of the effector. If there were no effect of the effector on the AR regions, this subpopulation would be expected to have the same affinity for DNA and RNA polymerase as does CO-bound CooA. This would result in a background of gene expression in the absence of CO that would be wasteful. However, the AR-positioning effect would presumably reduce the ability of this subpopulation to activate transcription in the absence of CO, even if it could bind DNA. The net effect of the phenomenon would be to decrease the level of activation in the absence of the effector, which in a sense increases the apparent CO specificity of the regulatory system.

Intersubunit communication in CooA. To this point, we have focused on the communication between the effector- and DNA-binding domains after CO binding. However, the proximity of the hemes to the shared C helices, and therefore to each other, makes communication between the two monomers a reasonable possibility. Such communication between monomers should also result from the fact that the Pro2 displaced by CO is from the other subunit. Intersubunit communication is also an obvious possibility for CRP, since each bound cAMP makes contacts with residues from both subunits. However, there has long been a disagreement about whether this communication causes cAMP binding to be positively or negatively cooperative. The current view is that binding is positively cooperative, and it appears that claims for negative cooperativity are probably based on the binding of two additional molecules of cAMP to the protein. These additional molecules bind to the DNA-binding domains themselves (99) and reduce the affinity of the protein for DNA, although the physiological significance of this form is doubtful.

The notion of communication between the hemes of CooA was first shown by the binding of CN^- to CooA variants altered at position 77 (124). Because of the perturbation of the normal His77 ligand, the CN^- binding in these cases is almost certainly on the opposite side of the heme relative to that bound by CO in wild-type CooA. Nevertheless, different substitutions showed different sorts of cooperative binding, demonstrating intersubunit communication in these variants. For CO binding to wild-type CooA, there are technical challenges presented by high CO affinity and low CO solubility. However, recent work by the laboratories of Spiro and Olson have now addressed the problem through kinetic and spectropscopic analyses (103). They have demonstrated that CooA is positively cooperative for CO binding and reflects the relative effect of a bound CO on the ability of CO and the other Pro2 ligand to compete for the heme iron. It appears that binding of CO to one heme in the dimer lowers the deligation rate of the Pro2 ligand to the other heme, which by itself would yield negative CO cooperativity. However, this CO binding also lowers the rebinding rate of the Pro2 to the other ligand by a much greater degree. The result is positive cooperativity for CO binding, because the other heme is now more accessible to CO. The biological implication of this cooperativity is that low levels of CO would activate a subpopulation of the CooA and therefore lead to transcription activation, even if the CO levels were insufficient to saturate the entire population of CooA hemes.

The situation in vivo is obviously more complex and has not yet been analyzed. For example, the interaction of CooA with DNA and RNA polymerase almost certainly affects the equilibrium between the active and inactive forms, which would have significant biological consequences.

FUTURE DIRECTIONS AND OPEN QUESTIONS

A major remaining question in the area of CO sensing is the physiological relevance of CO binding by sGC and the separate but related question of the biochemical basis for the CO effects seen in mammals. The small molecule that allows sGC to serve as a highly effective CO sensor in vitro is certainly tantalizing, but a physiologically relevant analog must be found if progress on the matter is to be made. Certainly, a challenging aspect of all work on sGC has been the difficulty in obtaining substantial amounts of highly active protein. This has been partially addressed by cloning the relevant functions into *E. coli*, but there are technical concerns about the similarity of this protein to that found in vivo. Should sGC prove not to be the basis of the observed CO effects, then there must be another CO sensor in mammals that has not yet been discovered.

The ability of certain microbes to perform aerobic CO oxidation strongly implies a basis of CO sensing. Based on both

the inappropriateness of CooA to function under such conditions and the apparent absence of *cooA* homologous sequences in these genomes, it appears that a rather different sensor is involved. This will doubtless be studied and understood in the foreseeable future.

For CooA, there remain the problems of our ignorance of the oxidized and CO-bound forms of the protein, which are critical for any complete understand of the transitions between the various protein forms. While progress has been made on the signal transduction system within the protein, substantial gaps in our knowledge remain. The actual basis for CO selectivity also remains unresolved. Finally, there are a host of physiological issues that have so far been ignored. These include the actual kinetics of the response of CooA to the appearance and then the elimination of CO in vivo. In part, this will require a better appreciation of the role of CooA interactions with DNA and RNA polymerase in altering critical biochemical parameters of the protein.

ACKNOWLEDGMENTS

This work was supported by the College of Agricultural and Life Sciences at the University of Wisconsin—Madison, National Institutes of Health grant GM53228 (to G.P.R.), and the Vilas Trust.

We thank Jose Serate and Mary Conrad for technical assistance throughout the work described and colleagues in the laboratories of Judith Burstyn, Tom Poulos, Tom Spiro, and John Olson for their intellectual input into and support of the work with CooA. Finally, we thank Rick Gourse, Tricia Kiley, Tim Donohue, Bob Landick, Tom Record, and members of their laboratories for discussions and advice about the general issues of protein-DNA interactions. Progress would also not have been possible without the work of previous members of the laboratory including Daniel Shelver, Marc Thorsteinsson, and Yiping He. Protein sequences shown in Fig. 4 were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Preliminary sequence data for *C. hydrogenoformans* and *D. vulgaris* were obtained from The Institute for Genomic Research website at http://www.tigr.org. Sequence data for the *E. coli* CRP protein are the copyrighted material of the Swiss Institute of Bioinformatics and the European Bioinformatics Institute (http://www.expasy.ch/sprot).

ADDENDUM IN PROOF

An analysis of the shape of CooA in solution as determined by small-angle X-ray scattering has recently been reported by S. Akiyama, T. Fujisawa, K. Ishimori, and S. Aono (J. Mol. Biol. **341:**651–668).

REFERENCES

1. **Abelsohn, A., M. D. Sanborn, B. J. Jessiman, and E. Weir.** 2002. Identifying and managing adverse environmental health effects. 6. Carbon monoxide poisoning. CMAJ **166:**1685–1690.
2. **Adams, M. W. W., and E. I. Stiefel.** 2000. Organometallic iron: the key to biological hydrogen metabolism. Curr. Opin. Chem. Biol. **4:**214–220.
3. **Akimoto, S., A. Tanaka, K. Nakamura, Y. Shiro, and H. Nakamura.** 2003. O_2-specific regulation of the ferrous heme-based sensor kinase FixL from *Sinorhizobium meliloti* and its aberrant inactivation in the ferric form. Biochem. Biophys. Res. Commun. **304:**136–142.
4. **Albracht, S. P. J., and R. Hedderich.** 2000. Learning from hydrogenases: location of a proton pump and of a second FMN in bovine NADH-ubiquinone oxidoreductase (complex I). FEBS Lett. **485:**1–6.
5. **Aono, S.** 2003. Biochemical and biophysical properties of the CO-sensing transcriptional activator CooA. Acc. Chem. Res. **36:**825–831.
6. **Aono, S., K. Ohkubo, T. Matsuo, and H. Nakajima.** 1998. Redox-controlled ligand exchange of the heme in the CO-sensing transcriptional activator *cooA*. J. Biol. Chem. **273:**25757–25764.
7. **Arendsen, A. F., M. Q. Soliman, and S. W. Ragsdale.** 1999. Nitrate-dependent regulation of acetate biosynthesis and nitrate respiration by *Clostridium thermoaceticum*. J. Bacteriol. **181:**1489–1495.
8. **Bell, A., and S. Busby.** 1994. Location and orientation of an activating region in the *Escherichia coli* transcription factor, FNR. Mol. Microbiol. **11:**383–390.
9. **Bell, A. I., K. L. Gaston, J. A. Cole, and S. J. W. Busby.** 1989. Cloning of binding sequences for the *Escherichia coli* transcription activators, FNR and CRP: location of bases involved in the discrimination between FNR and CRP. Nucleic Acids Res. **17:**3865–3874.
10. **Bernhard, M., T. Buhrke, B. Bleijlevens, A. L. De Lacey, V. M. Fernandez, S. P. Albracht, and B. Friedrich.** 2001. The H_2 sensor of *Ralstonia eutropha*. Biochemical characteristics, spectroscopic properties, and its interaction with a histidine protein kinase. J. Biol. Chem. **276:**15592–15597.
11. **Boehning, D., and S. H. Snyder.** 2002. Circadian rhythms. Carbon monoxide and clocks. Science **298:**2339–2340.
12. **Boehning, D., and S. H. Snyder.** 2003. Novel neural modulators. Annu. Rev. Neurosci. **26:**105–131.
13. **Bonam, D., S. A. Murrell, and P. W. Ludden.** 1984. Carbon monoxide dehydrogenase from *Rhodospirillum rubrum*. J. Bacteriol. **159:**693–699.
14. **Brouard, S., L. E. Otterbein, J. Anrather, E. Tobiasch, F. H. Bach, A. M. Choi, and M. P. Soares.** 2000. Carbon monoxide generated by heme oxygenase-1 suppresses endothelial cell apoptosis. J. Exp. Med. **192:**1015–1026.
15. **Brüne, B., and V. Ullrich.** 1987. Inhibition of platelet aggregation by carbon monoxide is mediated by activation of guanylate cyclase. Mol. Pharmacol. **32:**497–504.
16. **Busby, S., and R. H. Ebright.** 1999 Transcription activation by catabolite activator protein (CAP). J. Mol. Biol. **293:**199–213.
17. **Carr, H. S., D. Tran, M. F. Reynolds, J. N. Burstyn, and T. G. Spiro.** 2002. Demonstration of the role of scission of the proximal histidine-iron bond in the activation of soluble guanylyl cyclase through metalloporphyrin substitution studies. J. Am. Chem. Soc. **119:**7316–7323.
18. **Champagne, D. E., R. H. Nussenzveig, and J. M. Ribeiro.** 1995. Purification, partial characterization, and cloning of nitric oxide-carrying heme proteins (nitrophorins) from salivary glands of the blood-sucking insect *Rhodnius prolixus*. J. Biol. Chem. **270:**8691–8695.
19. **Chang, A. L., J. R. Tuckerman, G. Gonzalez, R. Meyer, H. Weinhouse, G. Volman, D. Amikam, M. Benziman, and M. A. Gilles-Gonzalez.** 2001. Phosphodiesterase A1, a regulator of cellulose synthesis in *Acetobacter xylinum*, is a heme-based sensor. Biochemistry **40:**3420–3426.
20. **Christiansen, J., L. C. Seefeldt, and D. R. Dean.** 2000. Competitive substrate and inhibitor interactions at the physiologically relevant active site of nitrogenase. J. Biol. Chem. **275:**36104–36107.
21. **Collier, J., and P. Vallance.** 1989. Second messenger role for NO widens to nervous and immune systems. Trends Pharmacol. Sci. **10:**427–431.
22. **Collman, J. P., J. I. Brauman, T. R. Halbert, and K. S. Suslick.** 1976. Nature of O_2 and CO binding to metalloporphyrins and heme proteins. Proc. Natl. Acad. Sci. USA **73:**3333–3337.
23. **Conrad, R.** 1996. Soil microorganisms as controllers of atmospheric trace gases. Microbiol. Rev. **60:**609–640.
24. **Coyle, C. M., M. Puranik, H. Youn, S. B. Nielsen, R. D. Williams, R. L. Kerby, G. P. Roberts, and T. G. Spiro.** 2003. Activation mechanism of the CO sensor CooA: Mutational and resonance Raman spectroscopic studies. J. Biol. Chem. **278:**35384–35393.
25. **Dai, Y., P. C. Wensink, and R. H. Abeles.** 1999. One protein, two enzymes. J. Biol. Chem. **274:**1193–1195.
26. **Darnault, C., A. Volbeda, E. J. Kim, P. Legrand, X. Vernède, P. A. Lindahl, and J. C. Fontecilla-Camps.** 2003. Ni-Zn-[Fe_4-S_4] and Ni-Ni-[Fe_4-S_4] clusters in closed and open α subunits of acetyl-CoA synthase/carbon monoxide dehydrogenase. Nat. Struct. Biol. **10:**271–279.
27. **Delgado-Nixon, V. M., G. Gonzalez, and M. A. Gilles-Gonzalez.** 2000. Dos, a heme-binding PAS protein from *Escherichia coli*, is a direct oxygen sensor. Biochemistry **39:**2685–2691.
28. **Dierks, E. A., S. Hu, K. M. Vogel, A. E. Yu, T. G. Spiro, and J. N. Burstyn.** 1997. Heme stoichiometry of heterodimeric soluble guanylate cyclase. Biochemistry **34:**14668–14674.
29. **Dioum, E. M., J. Rutter, J. R. Tuckerman, G. Gonzalez, M. A. Gilles-Gonzalez, and S. L. McKnight.** 2002. NPAS2: a gas-responsive transcription factor. Science **298:**2385–2387.
30. **Dobbek, H., L. Gremer, O. Meyer, and R. Huber.** 1999. Crystal structure and mechanism of CO dehydrogenase, a molybdo iron-sulfur flavoprotein containing *S*-selenocysteine. Proc. Natl. Acad. Sci. USA **96:**8884–8889.
31. **Dobbek, H., L. Gremer, R. Kiefersauer, R. Huber, and O. Meyer.** 2002. Catalysis at a dinuclear [CuSMo(=O)OH] cluster in a CO dehydrogenase resolved at 1.1-Å resolution. Proc. Natl. Acad. Sci. USA **99:**15971–15976.
32. **Dobbek, H., V. Svetlitchnyi, L. Gremer, R. Huber, and O. Meyer.** 2001. Crystal structure of a carbon monoxide dehydrogenase reveals a [Ni-4Fe-5S] cluster. Science **293:**1281–1285.
33. **Doukov, T. I., T. M. Iverson, J. Seravalli, S. W. Ragsdale, and C. L. Drennan.** 2002. A Ni-Fe-Cu center in a bifunctional carbon monoxide dehydrogenase/acetyl-CoA synthase. Science **298:**567–572.
34. **Drake, H. L.** 1994. Acetogenesis, acetogenic bacteria, and the acetyl-CoA "Wood/Ljungdahl" pathway: past and current perspectives, p. 3–60. *In* H. L. Drake (ed.), Acetogenesis. Chapman & Hall, New York, N.Y.
35. **Drennan, C. L., J. Heo, M. D. Sintchak, E. Schreiter, and P. W. Ludden.**

2001. Life on carbon monoxide: X-ray structure of *Rhodospirillum rubrum* Ni-Fe-S carbon monoxide dehydrogenase. Proc. Natl. Acad. Sci. USA **98:** 11973–11978.
36. **Dunham, C. M., E. M. Dioum, J. R. Tuckerman, G. Gonzalez, W. G. Scott, and M.-A. Gilles-Gonzalez.** 2003. A distal arginine in oxygen-sensing heme-PAS domains is essential to ligand binding, signal transduction, and structure. Biochemistry **42:**7701–7708.
37. **Ensign, S. A., and P. W. Ludden.** 1991. Characterization of the CO oxidation/H_2 evolution system of *Rhodospirillum rubrum*. J. Biol. Chem. **266:** 18395–18403.
38. **Esposti, M. D.** 1989. Prediction and comparison of the haem-binding sites in membrane haemoproteins. Biochim. Biophys. Acta. **977:**249–265.
39. **Ferry, J. G.** 1995. CO dehydrogenase. Annu. Rev. Microbiol. **49:**305–333.
40. **Ferry, J. G.** 1999. Enzymology of one-carbon metabolism in methanogenic pathways. FEMS Microbiol. Rev. **23:**13–38.
41. **Fox, J. D., R. L. Kerby, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the CO-induced, CO-tolerant hydrogenase from *Rhodospirillum rubrum* and the gene encoding the large subunit of the enzyme. J. Bacteriol. **178:**1515–1524.
42. **Fox, J. D., Y. He, D. Shelver, G. P. Roberts, and P. W. Ludden.** 1996. Characterization of the region encoding the CO-induced hydrogenase of *Rhodospirillum rubrum*. J. Bacteriol. **178:**6200–6208.
43. **Friebe, A., B. Wedel, C. Harteneck, J. Foerster, G. Schultz, and D. Koesling.** 1997. Functions of conserved cysteines of soluble guanylyl cyclase. Biochemistry **36:**1194–1198.
44. **Friebe, A., G. Schultz, and D. Koesling.** 1996. Sensitizing soluble guanylyl cyclase to become a highly CO-sensitive enzyme. EMBO J. **15:**6863–6868.
45. **Frey, M.** 2002. Hydrogenases: hydrogen-activating enzymes. Chembiochem **3:**153–160.
46. **Fujita, T., K. Toda, A. Karimova, S. F. Yan, Y. Naka, S. F. Yet, and D. J. Pinsky.** 2001. Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. Nat. Med. **7:**598–604.
47. **Gilles-Gonzalez, M. A., G. Gonzalez, and M. F. Perutz.** 1995. Kinase activity of oxygen sensor FixL depends on the spin state of its heme iron. Biochemistry **34:**232–236.
48. **Gilles-Gonzalez, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature **350:**170–172.
49. **Gnida, M., R. Ferner, L. Gremer, O. Meyer, and W. Meyer-Klaucke.** 2003. A novel binuclear [CuSMo] cluster at the active site of carbon monoxide dehydrogenase: characterization by X-ray absorption spectroscopy. Biochemistry **42:**222–230.
50. **Gong, W., G. Hao, and M. K. Chan.** 2000. New mechanistic insights from structural studies of the oxygen-sensing domain of *Bradyrhizobium japonicum* FixL. Biochemistry **39:**3955–3962.
51. **Gong, W., B. Hao, S. S. Mansy, G. Gonzalez, M. A. Gilles-Gonzalez, and M. K. Chan.** 1998. Structure of a biological oxygen sensor: a new mechanism for heme-driven signal transduction. Proc. Natl. Acad. Sci. USA **95:** 15177–15182.
52. **Grahame, D. A.** 2003. Acetate C—C bond formation and decomposition in the anaerobic world: the structure of a central enzyme and its key active-site metal cluster. Trends Biochem. Sci. **28:**221–224.
53. **Gremer, L., S. Kellner, H. Dobbek, R. Huber, and O. Meyer.** 2000. Binding of flavin adenine dinucleotide to molybdenum-containing carbon monoxide dehydrogenase from *Oligotropha carboxidovorans*. Structural and functional analysis of a carbon monoxide dehydrogenase species in which the native flavoprotein has been replaced by its recombinant counterpart produced in *Escherichia coli* J. Biol. Chem. **275:**1864–1872.
54. **Gunasekera, A., Y. W. Ebright, and R. H. Ebright.** 1992. DNA sequence determinants for binding of the *Escherichia coli* catabolite gene activator protein. J. Biol. Chem. **267:**14713–14720.
55. **Hänzelmann, P., H. Dobbek, L. Gremer, R. Huber, and O. Meyer.** 2000. The effect of intracellular molybdenum in *Hydrogenophaga pseudoflava* on the crystallographic structure of the seleno-molybdo-iron-sulfur flavoenzyme carbon monoxide dehydrogenase. J. Mol. Biol. **301:**1221–1235.
56. **Hao, B., C. Isaza, J. Arndt, M. Soltis, and M. K. Chan.** 2002. Structure-based mechanism of O_2 sensing and ligand discrimination by the FixL heme domain of *Bradyrhizobium japonicum*. Biochemistry. **41:**12952–12958.
57. **Harman, J. G.** 2001. Allosteric regulation of the cAMP receptor protein. Biochim. Biophys. Acta **1547:**1–17.
58. **He, Y., D. Shelver, R. L. Kerby, and G. P. Roberts.** 1996. Characterization of a CO-responsive transcriptional activator from *Rhodospirillum rubrum*. J. Biol. Chem. **271:**120–123.
59. **He, Y., T. Gaal, R. Karls, T. Donohue, R. Gourse, and G. P. Roberts.** 1999. Transcription activation by CooA, the CO-sensing factor from *Rhodospirillum rubrum*: the interaction between CooA and the C-terminal domain of the α subunit of RNA polymerase. J. Biol. Chem. **274:**10840–10846.
60. **Heo, J., C. M. Halbleib, and P. W. Ludden.** 2001. Redox-dependent activation of CO dehydrogenase from *Rhodospirillum rubrum*. Proc. Natl. Acad. Sci. USA **98:**7690–7693.
61. **Heo, J., C. R. Staples, C. M. Halbleib, and P. W. Ludden.** 2000. Evidence for a CO ligand in CO-dehydrogenase from *Rhodospirillum rubrum* that is required for catalytic activity. Biochemistry **39:**7956–7963.
62. **Heyduk, E., T. Heyduk, and J. C. Lee.** 1992. Global conformational changes in allosteric proteins. A study of *Escherichia coli* cAMP receptor protein and muscle pyruvate kinase. J. Biol. Chem. **267:**3200–3204.
63. **Hirsch, P.** 1968. Photosynthetic bacterium growing under carbon monoxide. Nature **217:**555–556.
64. **Huber, C., and G. Wächtershäuser.** 1997. Activated acetic acid by carbon fixation on (Fe, Ni)S under primordial conditions. Science **276:**245–247.
65. **Ignarro, L. J., K. S. Wood, and M. S. Wolin.** 1982. Activation of purified soluble guanylate cyclase by protoporphyrin IX. Proc. Natl. Acad. Sci. USA **79:**2870–2873.
66. **Ingi, T., and G. V. Ronnett.** 1995. Direct demonstration of a physiological role for carbon monoxide in olfactory receptor neurons. J. Neurosci. **15:** 8214–8222.
67. **Jain, R., and M. K. Chan.** 2003. Mechanisms of ligand discrimination by heme proteins. J. Biol. Inorg. Chem. **8:**1–11.
68. **Jeon, W. B., J. Cheng, and P. W. Ludden.** 2001. Purification and characterization of membrane-associated CooC protein and its functional role in the insertion of nickel into carbon monoxide dehydrogenase from *Rhodospirillum rubrum*. J. Biol. Chem. **276:**38602–38609.
69. **Joshi, H. M., and F. R. Tabita.** 2000. Induction of carbon monoxide dehydrogenase to facilitate redox balancing in a ribulose bisphosphate carboxylase/oxygenase-deficient mutant strain of *Rhodospirillum rubrum*. Arch. Microbiol. **173:**193–199.
70. **Kamisaki, Y., S. Saheki, M. Nakane, J. A. Palmieri, T. Kuno, B. Y. Chang, S. A. Waldman, and F. Murad.** 1986. Soluble guanylate cyclase from rat lung exists as a heterodimer. J. Biol. Chem. **261:**7236–7241.
71. **Kerby, R. L., H. Youn, M. V. Thorsteinsson, and G. P. Roberts.** 2003. Repositioning about the dimer interface of the transcription regulator CooA: A major signal transduction pathway between the effector- and DNA-binding domains. J. Mol. Biol. **325:**809–823.
72. **Kerby, R., and J. G. Zeikus.** 1987. Catabolic enzymes of the acetogen *Butyribacterium methylotrophicum* grown on single-carbon substrates. J. Bacteriol. **169:**5605–5609.
73. **Kerby, R. L., P. W. Ludden, and G. P. Roberts.** 1997. In vivo nickel insertion into the carbon monoxide dehydrogenase of *Rhodospirillum rubrum*: molecular and physiological characterization of *cooCTJ*. J. Bacteriol. **179:** 2259–2266.
74. **Kerby, R. L., S. S. Hong, S. A. Ensign, L. J. Coppoc, P. W. Ludden, and G. P. Roberts.** 1992. Genetic and physiological characterization of the *Rhodospirillum rubrum* carbon monoxide dehydrogenase system. J. Bacteriol. **174:**5284–5294.
75. **Kharitonov, V. G., V. S. Sharma, D. Magde, and D. Koesling.** 1999. Kinetics and equilibria of soluble guanylate cyclase ligation by CO: effect of YC-1. Biochemistry **38:**10699–10706.
76. **Khoroshilova, N., H. Beinert, and P. J. Kiley.** 1995. Association of a polynuclear iron-sulfur center with a mutant FNR protein enhances DNA binding. Proc. Natl. Acad. Sci. USA **92:**2499–2503.
77. **Kiley, P. J., and H. Beinert.** 2003. The role of Fe-S proteins in sensing and regulation in bacteria. Curr. Opin. Microbiol. **6:**181–185.
78. **Kim, Y. M., and G. D. Hegeman.** 1983. Oxidation of carbon monoxide by bacteria. Int. Rev. Cytol. **81:**1–32.
79. **Kolb, A., S. Busby, H. Buc, S. Garges, and S. Adhya.** 1993. Transcriptional regulation by cAMP and its receptor protein. Annu. Rev. Biochem. **62:**749–795.
80. **Kumar, S. A., N. S. Murthy, and J. S. Krakow.** 1980. Ligand-induced change in the radius of gyration of cAMP receptor protein from *Escherichia coli*. FEBS Lett. **109:**121–124.
81. **Lanzilotta, W. N., D. J. Schuller, M. V. Thorsteinsson, R. L. Kerby, G. P. Roberts, and T. L. Poulos.** 2000. Structure of the CO sensing transcription activator CooA. Nat. Struct. Biol. **7:**876–880.
82. **Leduc, J., M. V. Thorsteinsson, T. Gaal, and G. P. Roberts.** 2001. Mapping CooA-RNA polymerase interactions: identification of activating regions 2 & 3 in CooA, the CO-sensing transcriptional activator. J. Biol. Chem. **276:**39968–39973.
83. **Lee, H.-I., L. M. Cameron, B. J. Hales, and B. M. Hoffman.** 1997. CO binding to the FeMo cofactor of CO-inhibited nitrogenase: ^{13}CO and ^{1}H Q-band ENDOR investigation. J. Am. Chem. Soc. **119:**10121–10126.
84. **Lonetto, M. A., V. Rhodius, K. Lamberg, P. Kiley, S. Busby, and C. Gross.** 1998. Identification of a contact site for different transcription activators in region 4 of the *Escherichia coli* RNA polymerase σ^{70} subunit. J. Mol. Biol. **284:**1353–1365.
85. **Mason, W. P.** 1888. Fatal poisoning by carbon monoxide. J. Am. Chem. Soc. **10:**176–178.
86. **Maynard, E. L., and P. A. Lindahl.** 1999. Evidence of a molecular tunnel connecting the active sites for CO_2 reductions and acetyl-CoA synthesis in acetyl-CoA synthase from *Clostridium thermoaceticum*. J. Am. Chem. Soc. **121:**9221–9222.
87. **Meyer, O., L. Gremer, R. Ferner, M. Ferner, H. Dobbek, M. Gnida, W. Meyer-Klaucke, and R. Huber.** 2000. The role of Se, Mo and Fe in the

structure and function of carbon monoxide dehydrogenase. Biol. Chem. **381:**865–876.
88. **Meyer, O., S. Jacobitz, and B. Krüger.** 1986. Biochemistry and physiology of aerobic carbon monoxide-utilizing bacteria. FEMS Microbiol. Rev. **39:** 161–179.
89. **Miyakawa, S., H. Yamanashi, K. Kobayashi, H. J. Cleaves, and S. L. Miller.** 2002. Prebiotic synthesis from CO atmospheres: implications for the origins of life. Proc. Natl. Acad. Sci. USA **99:**14628–14631.
90. **Monson, R. K., and E. A. Holland.** 2001. Biospheric trace gas fluxes and their control over tropospheric chemistry. Annu. Rev. Ecol. Syst. **32:**547–576.
91. **Mörsdorf, G., K. Frunzke, D. Gadkari, and O. Meyer.** 1992. Microbial growth on carbon monoxide. Biodegradation **3:**61–82.
92. **Mott, J. A., M. I. Wolfe, C. J. Alverson, S. C. Macdonald, C. R. Bailey, L. B. Ball, J. E. Moorman, J. H. Somers, D. M. Mannino, and S. C. Redd.** 2002. National vehicle emissions policies and practices and declining US carbon monoxide-related mortality. JAMA **288:**988–995.
93. **Nakajima, H., E. Nakagawa, K. Kobayashi, S. Tagawa, and S. Aono.** 2001. Ligand-switching intermediates for the CO-sensing transcriptional activator CooA measured by pulse radiolysis. J. Biol. Chem. **276:**37895–37899.
94. **Nakajima, H., and S. Aono.** 1999. Electrochemical evidence of the redox-controlled ligand exchange of the heme in the CO-sensing transcriptional activator CooA. Chem. Lett. **1999:**1233–1234.
95. **Niu, W., Y. Kim, G. Tau, T. Heyduk, and R. H. Ebright.** 1996. Transcription activation at class II CAP-dependent promoters: two interactions between CAP and RNA polymerase. Cell **87:**1123–1134.
96. **Niu, W., Y. Zhou, Q. Dong, Y. Ebright, and R. H. Ebright.** 1994. Characterization of the activating region of *Escherichia coli* catabolite gene activator protein (CAP). I. Saturation and alanine-scanning mutagenesis. J. Mol. Biol. **243:**595–602.
97. **Otterbein, L. E., F. H. Bach, J. Alam, M. Soares, H. T. Lu, M. Wysk, R. J. Davis, R. A. Flavell, and A. M. Choi.** 2000. Carbon monoxide has anti-inflammatory effects involving the mitogen-activated protein kinase pathway. Nat. Med. **6:**422–428.
98. **Parkinson, G., C. Wilson, A. Gunasekera, Y. Ebright, R. Ebright, and H. Berman.** 1996. Structure of the CAP-DNA complex at 2.5 Å resolution. J. Mol. Biol. **260:**395–408.
99. **Passner, J. M., and T. A. Steitz.** 1997. The structure of a CAP-DNA complex having two cAMP molecules bound to each monomer. Proc. Natl. Acad. Sci. USA **94:**2843–2847.
100. **Passner, J. M., S. C. Schultz, and T. A. Steitz.** 2000. Modeling the cAMP-induced allosteric transition using the crystal structure of CAP-cAMP at 2.1 Å resolution. J. Mol. Biol. **304:**847–859.
101. **Pastan, I., and S. Adhya.** 1976. Cyclic adenosine 5′-monophosphate in *Escherichia coli*. Bacteriol. Rev. **40:**527–551.
102. **Pomposiello, P. J., and B. Demple.** 2001. Redox-operated genetic switches: the SoxR and OxyR transcription factors. Trends Biotechnol. **19:**109–114.
103. **Puranik, M., S. B. Nielsen, H. Youn, A. N. Hvitved, J. L. Bourassa, M. A. Case, C. Tengroth, G. Balakrishnan, M. V. Thorsteinsson, J. T. Groves, G. L. McLendon, G. P. Roberts, J. S. Olson, and T. G. Spiro.** 2004. Dynamics of carbon monoxide binding to CooA. J. Biol. Chem. **279:**21096–21108.
104. **Ragsdale, S. W., and M. Kumar.** 1996. Nickel-containing carbon monoxide dehydrogenase/acetyl-CoA synthase. Chem. Rev. **96:**2515–2539.
105. **Ramos, K. S., H. Lin, and J. J. McGrath.** 1989. Modulation of cyclic guanosine monophosphate levels in cultured aortic smooth muscle cells by carbon monoxide. Biochem. Pharmacol. **38:**1368–1370.
106. **Rees, D. C.** 2002. Great metalloclusters in enzymology. Annu. Rev. Biochem. **71:**221–246.
107. **Reynolds, M. F., D. Shelver, R. L. Kerby, R. B. Parks, G. P. Roberts, and J. N. Burstyn.** 1998. EPR and electronic absorption spectroscopies of the CO-sensing CooA protein reveal a cysteine-ligated low-spin heme. J. Am. Chem. Soc. **120:**9080–9081.
108. **Reynolds, M. F., R. B. Parks, J. N. Burstyn, D. Shelver, M. V. Thorsteinsson, R. L. Kerby, G. P. Roberts, K. M. Vogel, and T. G. Spiro.** 2000. Electronic absorption, EPR, and resonance Raman spectroscopy of CooA, a CO-sensing transcription activator from *R. rubrum*, reveals a five-coordinate NO-heme. Biochemistry **39:**388–396.
109. **Rhodius, V. A., and S. J. W. Busby.** 2000. Transcription activation by the *Escherichia coli* cyclic AMP receptor protein: determinants within activating region 3. J. Mol. Biol. **299:**295–310.
110. **Ribbe, M., D. Gadkari, and O. Meyer.** 1997. N_2 fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple N_2 reduction to the oxidation of superoxide produced from O_2 by a molybdenum-CO dehydrogenase. J. Biol. Chem. **272:**26627–26633.
111. **Roberts, G. P., M. V. Thorsteinsson, R. L. Kerby, W. N. Lanzilotta, and T. L. Poulos.** 2001. CooA: a heme-containing regulatory protein that serves as a specific sensor of both carbon monoxide and redox state. Prog. Nucleic Acid Res. Mol. Biol. **67:**35–63.
112. **Rubtsov, I. V., T. Zhang, H. Nakajima, S. Aono, G. I. Rubtsov, S. Kumazaki, and K. Yoshihara.** 2001. Conformational dynamics of the transcriptional regulator CooA protein studied by subpicosecond mid-infrared vibrational spectroscopy. J. Am. Chem. Soc. **123:**10056–10062.
113. **Santiago, B., U. Schübel, C. Egelseer, and O. Meyer.** 1999. Sequence analysis, characterization and CO-specific transcription of the *cox* gene cluster on the megaplasmid pHCG3 of *Oligotropha carboxidovorans*. Gene **236:** 115–124.
114. **Scott, C., and J. Green.** 2002. Miscoordination of the iron-sulfur clusters of the anaerobic transcription factor, FNR, allows simple repression but not activation. J. Biol. Chem. **277:**1749–1754.
115. **Seravalli, J., and S. R. Ragsdale.** 2000. Channeling of carbon monoxide during anaerobic carbon dioxide fixation. Biochemistry **39:**1274–1277.
116. **Shelver, D., M. F. Reynolds, M. V. Thorsteinsson, R. L. Kerby, S.-Y. Chung, R. L. Parks, J. N. Burstyn, and G. P. Roberts.** 1999. Identification of two important heme site residues (Cys^{75} and His^{77}) in CooA, the CO-sensing transcription factor of *Rhodospirillum rubrum*. Biochemistry **38:**2669–2678.
117. **Shelver, D., R. L. Kerby, Y. He, and G. P. Roberts.** 1997. CooA, a CO-sensing transcription factor from *Rhodospirillum rubrum*, is a CO-binding heme protein. Proc. Natl. Acad. Sci. USA **94:**11216–11220.
118. **Soboh, B., D. Linder, and R. Hedderich.** 2002. Purification and catalytic properties of a CO-oxidizing:H_2-evolving enzyme complex from *Carboxydothermus hydrogenoformans*. Eur. J. Biochem. **269:**5712–5721.
119. **Spiro, T. G., and A. A. Jarzecki.** 2001. Heme-based sensors: theoretical modeling of heme-ligand-protein interactions. Curr. Opin. Chem. Biol. **5:**715–723.
120. **Stone, J. R., and M. A. Marletta.** 1994. Soluble guanylate cyclase from bovine lung: activation with nitric oxide and carbon monoxide and spectral characterization of the ferrous and ferric states. Biochemistry **33:**5636–5640.
121. **Svetlichny, V. A., T. G. Sokolova, M. Gerhardt, M. Ringpfeil, N. A. Kostrikina, and G. A. Zavarzin.** 1991. *Carboxydothermus hydrogenoformans* gen. nov., sp. nov., a CO-utilizing thermophilic anaerobic bacterium from hydrothermal environments of Kunashir island. Syst. Appl. Microbiol. **14:**254–260.
122. **Taylor, B. L., and I. B. Zhulin.** 1999. PAS domains: internal sensors of oxygen, redox potential and light. Microbiol. Mol. Biol. Rev. **63:**479–506.
123. **Thauer, R. K., G. Fuchs, B. Käufer, and U. Schnitker.** 1974. Carbon-monoxide oxidation in cell-free extracts of *Clostridium pasteurianum*. Eur. J. Biochem. **45:**343–349.
124. **Thorsteinsson, M. V., R. L. Kerby, and G. P. Roberts.** 2000. Altering the specificity of CooA, the carbon monoxide-sensing transcriptional activator: characterization of CooA variants that bind cyanide in the Fe^{II} form with high affinity. Biochemistry **39:**8284–8290.
125. **Thorsteinsson, M. V., R. L. Kerby, M. Conrad, H. Youn, C. R. Staples, W. N. Lanzilotta, T. J. Poulos, J. Serate, and G. P. Roberts.** 2000. Characterization of variants altered at the N-terminal proline, a novel heme-axial ligand in CooA, the CO-sensing transcriptional factor. J. Biol. Chem. **276:** 39332–39338.
126. **Tomita, T., G. Gonzalez, A. L. Chang, M. Ikeda-Saito, and M. A. Gilles-Gonzalez.** 2002. A comparative resonance Raman analysis of heme-binding PAS domains: heme iron coordination structures of the BjFixL, AxPDEA1, EcDos, and MtDos proteins. Biochemistry **41:**4819–4826.
127. **Tosques, I. E., J. Shi, and J. P. Shapleigh.** 1996. Cloning and characterization of *nnrR*, whose product is required for the expression of proteins involved in nitric oxide metabolism in *Rhodobacter sphaeroides* 2.4.3. J. Bacteriol. **178:**4958–4964.
128. **Tuckerman, J. R., G. Gonzalez, E. M. Dioum, and M. A. Gilles-Gonzalez.** 2002. Ligand and oxidation-state specific regulation of the heme-based oxygen sensor FixL from *Sinorhizobium meliloti*. Biochemistry **41:**6170–6177.
129. **Uchida, T., H. Ishikawa, K. Ishimori, I. Morishima, H. Nakajima, S. Aono, Y. Mizutani, and T. Kitagawa.** 2000. Identification of histidine 77 as the axial heme ligand of carbonmonoxy CooA by picosecond time-resolved resonance Raman spectroscopy. Biochemistry **39:**12747–12752.
130. **Uchida, T., H. Ishikawa, S. Takahashi, K. Ishimori, I. Morishima, K. Ohkubo, H. Nakajima, and S. Aono.** 1998. Heme environmental structure of CooA is modulated by the target DNA binding. Evidence from resonance Raman spectroscopy and CO rebinding kinetics J. Biol. Chem. **273:** 19988–19992.
131. **Uffen, R. L.** 1976. Anaerobic growth of a *Rhodopseudomonas* species in the dark with carbon monoxide as sole carbon and energy substrate. Proc. Natl. Acad. Sci. USA **73:**3298–3302.
132. **Uffen, R. L.** 1981. Metabolism of carbon monoxide. Enzyme Microb. Technol. **3:**197–206.
133. **Utz, J., and V. Ullrich.** 1991. Carbon monoxide relaxes ileum smooth muscle through activation of guanylate cyclase. Biochem. Pharmacol. **41:** 1195–1201.
134. **Varon, J., P. E. Marik, R. E. Fromm, and A. Gueler.** 1999. Carbon monoxide poisoning: a review for clinicians. J. Emerg. Med. **17:**87–93.
135. **Verma, A., D. J. Hirsch, C. E. Glatt, G. V. Ronnett, and S. H. Snyder.** 1993. Carbon monoxide: a putative neural messenger. Science **259:**381–384.
136. **Vincent, S. R., S. Das, and M. D. Maines.** 1994. Brain heme oxygenase

isoenzymes and nitric oxide synthase are co-localized in select neurons. Neuroscience **63:**223–231.

137. **Voordouw, G.** 2002. Carbon monoxide cycling by *Desulfovibrio vulgaris* Hildenborough. J. Bacteriol. **184:**5903–5911.
138. **Watt, R. K., and P. W. Ludden.** 1999. Ni^{2+} transport and accumulation in *Rhodospirillum rubrum*. J. Bacteriol. **181:**4554–4560.
139. **Weber, I. T., and T. A. Steitz.** 1987. Structure of a complex of catabolite gene activator protein and cyclic AMP refined at 2.5 Å resolution. J. Mol. Biol. **198:**311–326.
140. **Williams, R., A. Bell, G. Sims, and S. Busby.** 1991. The role of two surface exposed loops in transcription activation by the *Escherichia coli* CRP and FNR proteins. Nucleic Acids Res. **19:**6705–6712.
141. **Won, H.-S., T. Yamazaki, T.-W. Lee, M.-K. Yoon, S.-H. Park, Y. Kyogoku, and B.-J. Lee.** 2000. Structural understanding of the allosteric conformational change of cyclic AMP receptor protein by cyclic AMP binding. Biochemistry **39:**13953–13962.
142. **Wood, H. G., and L. G. Ljungdahl.** 1991. Autotrophic character of the acetogenic bacteria, p. 201–250. *In* J. M. Shively and L. L. Barton (ed.), Variations in autotrophic life. Academic Press Inc., San Diego, Calif.
143. **Yagi, T.** 1958. Enzymic oxidation of carbon monoxide. Biochim. Biophys. Acta. **30:**194–195.
144. **Yamamoto, K., H. Ishikawa, S. Takahashi, K. Ishimori, I. Morishima, H. Nakajima, and S. Aono.** 2001. Binding of CO at the Pro2 side is crucial for the activation of CO-sensing transcriptional activator CooA. ^{1}H NMR spectroscopic studies. J. Biol. Chem. **276:**11473–11476.
145. **Yamashita, T., Y. Hoashi, K. Watanabe, Y. Tomisugi, Y. Ishikawa, and T. Uno.** 2004. Roles of heme axial ligands in the regulation of CO binding to CooA. J. Biol. Chem. **279:**21394–21400.
146. **Youn, H., R. L. Kerby, and G. P. Roberts.** 2003. The role of the hydrophobic distal heme pocket of CooA in ligand sensing and response. J. Biol. Chem. **278:**2333–2340.
147. **Youn, H., R. L. Kerby, M. Conrad, and G. P. Roberts.** 2004. Functionally critical elements of CooA-related CO sensors. J. Bacteriol. **186:**1320–1329.
148. **Youn, H., R. L. Kerby, M. V. Thorsteinsson, M. Conrad, C. R. Staples, J. Serate, J. Beack, and G. P. Roberts.** 2001. The heme pocket afforded by Gly^{117} is crucial for proper heme ligation and activity of CooA. J. Biol. Chem. **276:**41603–41610.
149. **Youn, H., R. L. Kerby, M. V. Thorsteinsson, M. Conrad, R. W. Clarke, J. N. Burstyn, and G. P. Roberts.** 2002. Analysis of a variant of CooA, the heme-containing CO sensor, suggests the presence of an unusual heme ligand results in novel activity. J. Biol. Chem. **277:**33616–33623.
150. **Zhao, Y., P. E. Brandish, D. P. Ballou, and M. A. Marletta.** 1999. A molecular basis for nitric oxide sensing by soluble guanylate cyclase. Proc. Natl. Acad. Sci. USA **96:**14753–14758.
151. **Zumft, W. G.** 2002. Nitric oxide signaling and NO dependent transcriptional control in bacterial denitrification by members of the FNR-CRP regulator family. J. Mol. Microbiol. Biotechnol. **4:**277–286.