Positively Charged Amino Acids Are Essential for Electron Transfer and Protein−Protein Interactions in the Soluble Methane Monooxygenase Complex from *Methylococcus capsulatus* (Bath)†

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ABSTRACT: The soluble methane monooxygenase (sMMO) complex from *Methylococcus capsulatus* (Bath) catalyses oxygen- and NAD(P)H-dependent oxygenation of methane, propene, and other substrates. Whole-complex sMMO oxygenase activity requires all three sMMO components: the hydroxylase, the reductase, and protein B. Also, in the presence of hydrogen peroxide, the hydroxylase alone catalyzes substrate oxygenation via the peroxide shunt reaction. We investigated the effect of amine cross-linking on hydroxylase activity to probe the role of a gross conformational change that occurs in the hydroxylase upon binding of the other protein components. The cross-linker inhibited hydroxylase activity in the whole complex, but this effect was due to covalent modification of primary amine groups rather than cross-linking. Covalent modification of arginine side-chains on the hydroxylase had a similar effect, but, most remarkably, neither form of modification affected the activity of the hydroxylase via the peroxide shunt reaction. It was shown that covalent modification of positively charged groups on the hydroxylase, which occurred at multiple sites, interfered with its physical and functional interactions with protein B and with the passage of electrons from the reductase. These results indicate that protein B and the reductase of the sMMO complex interact via positively charged groups on the surface of the hydroxylase to induce a conformational change that is necessary for delivery of electrons into the active site of the hydroxylase. Modification of positively charged groups on protein B had no effect on its function, consistent with the hypothesis that positively charged groups on the hydroxylase interact with negative charges on protein B. Thus, we have discovered a means of specifically inactivating the interactions between the sMMO complex while preserving the catalytic activity of the hydroxylase active site which provides a new method of studying intercomponent interactions within sMMO.

Methanotrophic bacteria such as *Methylococcus capsulatus* (Bath) can grow using methane as sole carbon and energy source and possess a multistep metabolic pathway to oxidize methane to carbon dioxide (*1*, *2*). The initial step in the methane oxidation pathway is the conversion of methane to methanol, catalyzed under conditions of copper-limiting growth by soluble methane monooxygenase (sMMO)[1] (*3*) in an NADH- and O_2-dependent reaction.

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+$$

In addition to its natural substrate methane, sMMO also oxygenates a range of other substrates including alkanes, alkenes, aromatics, and chlorinated hydrocarbons (*4*−*6*). sMMO consists of three components: a hydroxylase, a reductase, and a regulatory component termed protein B (*7*, *8*). The X-ray structure of the hydroxylase shows that it exists as a 251-kDa $(\alpha\beta\gamma)_2$ dimer. Each α subunit contains a μ-(hydr)oxo-bridged binuclear iron species in a hydrophobic cleft that is thought to be the site of methane and oxygen activation (*9*−*12*). The 38-kDa reductase contains a [2Fe-2S] cluster and an FAD group. It transfers electrons from NADH to the binuclear iron active site of the hydroxylase via its two prosthetic groups (*13*, *14*). The 16-kDa cofactorless protein B is necessary for the full activity of the sMMO complex and influences the redox potentials of the binuclear iron center in the hydroxylase (*8*, *15*, *16*), substrate specificity, and product distribution (*17*). It also promotes electron transfer (*18*), couples electron transfer to substrate oxygenation (*13*), and accelerates formation of the high-valent intermediate Q, which is the form of the hydroxylase thought to be responsible for substrate oxygenation (*19*).

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[1] Abbreviations: BS^3, bis(sulfosuccinimidyl) suberate; EDC, (1-ethyl-3-dimethylamino)-carbodiimide; GC, gas chromatography; GST, glutathione *S*-tranferase; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MOPS, 3-(*N*-morpholino) propanesulfonic acid; RU, response unit; SAXS, small-angle X-ray scattering spectroscopy; sMMO, soluble methane monooxygenase; SPR, surface plasmon resonance; sulfo-NHS-acetate, sulfosuccinimidyl acetate.

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The intricate involvement of all three components in catalysis by sMMO shows that protein−protein interactions are a crucial part of the system. Cross-linking studies using 1-ethyl-3-diethylamino-carbodiimide (EDC) have suggested that, in the homologous sMMO of *Methylosinus trichosporium* OB3b, the reductase binds to the β subunit of the hydroxylase and protein B binds to the α subunit, near an interface between the α and β subunits (*20*). Protein B induces a conformational change that perturbs the binuclear iron active site, as has been shown by electron paramagnetic resonance, circular dichroism, and fluorescence spectroscopy (*21*−*24*). Crystal structure data for the hydroxylase of *Mc. capsulatus* (Bath) have allowed a binding site for protein B to be proposed in the canyon formed between the $\alpha\beta$ pairs of the protein (*9*). Modeling studies, to dock the nuclear magnetic resonance-derived structure of protein B into its proposed binding site on the hydroxylase, showed that the central core of protein B could be easily positioned into this region and its mainly hydrophobic surface was well suited for binding at this site (*9*, *25*). Experimental evidence for such a hydrophobic interaction was observed when a degradation-resistant mutant of protein B was immobilized on a column for purification of the hydroxylase. It was found that binding of the hydroxylase was strengthened by NaCl, suggesting a primarily hydrophobic interaction between the two proteins (*26*).

There are currently no crystallographic data for the whole sMMO complex to indicate precisely how the proteins interact with one another. However, low-resolution structural data for the sMMO complex have been obtained by using small-angle X-ray scattering spectroscopy (SAXS). This technique has shown that protein B and the reductase induce a gross overall conformational change in the hydroxylase, whereby the two $\alpha\beta\gamma$ protomers rotate relative to one another and so the hydroxylase becomes a longer, thinner molecule (*27*).

It is possible to investigate the catalytic roles of protein−protein interactions in the sMMO complex because, even in the absence of protein B, the reductase, NADH, and oxygen, the hydroxylase can be activated by hydrogen peroxide to perform most of the oxygenation reactions catalyzed by the complete system (*28*). This reaction, which is known as the peroxide shunt, was originally observed in the functionally related monooxygenase cytochrome P450 (*29*−*31*). Interestingly, in the sMMO system, SAXS revealed no significant conformational change in the hydroxylase upon addition of hydrogen peroxide (*27*), suggesting that the conformational change observed on addition of the other protein components was not necessary for catalysis or was required only when electrons were delivered via the reductase. In light of the significant structural effect of the protein B and the reductase on the hydroxylase conformation, it is puzzling to note that the hydroxylase can function alone in the peroxide shunt without major structural reorganization.

In the present study, we wished to investigate the roles that protein B and the reductase play in sMMO catalysis through the conformational change that they induce in the hydroxylase. Since the SAXS study (*27*) suggested that the $\alpha\beta\gamma$ protomers rotate relative to one another by as much as 180°, we reasoned that it may be possible to block the conformational change by cross-linking the hydroxylase subunits. We therefore began our investigation of the roles of protein−protein interactions in the sMMO complex by studying the effects of a cross-linking agent on the hydroxylase component.

MATERIALS AND METHODS

Chemicals. Bis(sulfosuccinimidyl) suberate (BS^3), sulfo-succinimidyl acetate (sulfo-NHS-acetate), and *p*-hydroxyphenylglyoxal were purchased from Pierce. Propene was purchased from BOC Special Gases, epoxypropane from BDH, and hydrogen peroxide from Aldrich. NADH was obtained from Sigma, and residual ethanol was removed as previously described (*32*).

Purification of the sMMO Components. The hydroxylase and reductase components of sMMO were purified from *Mc. capsulatus* (Bath) as previously described (*32*). The protein B used in all experiments was a catalytically active mutant with increased stability, in which glycine 13 was replaced by glutamine. This recombinant protein was expressed as a fusion to glutathione *S*-transferase (GST) in *Escherichia coli*; it was purified as previously reported and, except where otherwise stated, the N-terminal GST tag was removed (*33*). Hydroxylase and protein B concentrations were determined at A_{280}. The molar extinction coefficients of these two proteins were experimentally determined to be 551 000 and 16 800 M^{-1} cm^{-1}, respectively [for methods see refs *34* and *35*]. These values were in good agreement with those previously determined (*18*, *27*) and are also in agreement with extinction coefficients deduced from the sequences of the two proteins (583 640 and 17 780 M^{-1} cm^{-1}, for the hydroxylase and the protein B, respectively). The concentration of the reductase was determined colorimetrically using the dye-binding method of Bradford (*36*).

Enzyme Assays. sMMO assays were performed using propene as the substrate. Reactions had a final volume of 100 μL and were incubated at 45 °C with shaking in 2-mL sealed reaction vials. Headspace gas (1 mL) was removed and replaced with 1 mL of propene, and epoxypropane (propene oxide) formation was measured after 3 min incubation by gas chromatography (GC) of 0.5 mL of the headspace gas using a Porapak Q column, as described previously (*28*). No epoxypropane peak was detected at time zero.

To measure the activity of the whole sMMO complex, hydroxylase (8 μM final concentration), regulatory protein B, and reductase were mixed at a molar ratio of 1:2:2 and preincubated at 45 °C with propene for 30 s. NADH was added to a final concentration of 10 mM, and epoxypropane formation was assayed after a further 3 min incubation as described above. For measurement of hydroxylase activity via the peroxide shunt, hydroxylase (20 μM), with or without regulatory protein B (100 μM), was preincubated with propene for 1 min. Hydrogen peroxide was added to a final concentration of 250 mM, and then after a further 2 min incubation at 45 °C, the epoxypropane formed was assayed by GC.

NADH oxidase activity was determined by measuring the decrease in absorbance due to NADH at 340 nm [$\epsilon = 6.22 \times 10^3$ M^{-1} cm^{-1} (*13*)] using a Hewlett-Packard 8452A spectrophotometer. Reaction mixtures (total volume 1 mL) contained 25 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH 7.0, with the following amounts of protein components in various combinations: hydroxylase (2 μM), protein B (1 μM), and reductase (4 μM). Propene, where

present, was at the same partial pressure in the gas phase as used for the GC assays. Reactions were initiated by adding NADH to 0.16 mM.

Covalent Modification of Positively Charged Groups. All covalent modification procedures were performed in 25 mM MOPS, pH 7. Cross-linking and neutralization of primary amine groups on the hydroxylase were achieved by reacting hydroxylase (76 μM final concentration) with BS^3 (cross-linker) or sulfo-NHS-acetate (neutralization reagent) at a modification reagent/hydroxylase monomer ($\alpha\beta\gamma$) molar ratio of 13:1. The reaction was incubated for 30 min at room temperature, and then the unreacted cross-linker or blocking reagent was removed by ultrafiltration using a 50-kDa cut off Microcon centrifugal filter (Amicon). To ensure adequate removal of the reagent, the ultrafiltration step was repeated twice, each time after addition of a new volume (equivalent to the initial volume of the sample) of 25 mM MOPS, pH 7.0. As a control, to confirm removal of the cross-linking or blocking reagent, a sample containing the buffer and the BS^3 or sulfo-NHS-acetate but no hydroxylase was subjected to identical incubation and buffer-exchange procedures. When the resulting solution was added to unreacted hydroxylase, activities in the whole sMMO complex and the peroxide shunt were not affected. Primary amine groups on protein B were neutralized exactly as on the hydroxylase, except that 8 μM of protein and a sulfo-NHS-acetate/protein molar ratio of 15:1 were used and the centrifugal concentrator used for the ultrafiltration procedure had a 10-kDa cutoff.

Modification of arginyl residues was performed by using *p*-hydroxyphenylglyoxal solution (at 12 mM final concentration), which was preincubated at 30 °C for 15 min before addition of the hydroxylase or protein B (to 40 μM). The reaction was incubated at 30 °C for a further 20 min before quenching with arginine (to 60 mM final concentration). Any unreacted *p*-hydroxyphenylglyoxal was removed by the appropriate ultrafiltration protocol described above.

Electrophoresis. Nondenaturing and SDS−PAGE were performed with a MiniProtean II gel apparatus (BioRad).

Surface Plasmon Resonance. Surface plasmon resonance (SPR) measurements were acquired at 25 °C using a BIAcore 2000 apparatus fitted with a CM5 sensor chip (BIAcore). Proteins were applied to the chip in HBS-EP buffer (150 mM NaCl, 3 mM EDTA, and 10 mM *N*-[2-hydroxyethyl]-piperazine-*N*′-[2-ethanesulfonic acid] [HEPES], pH 7.4) (BIAcore). Initially, anti-GST monoclonal antibody was immobilized on the chip by amine coupling according to the manufacturer's instructions. GST-tagged protein B was diluted to a concentration of 1 μg mL^{-1} in HBS-EB buffer, and then 30 μL of the resulting solution was injected onto the chip at a flow rate of 5 μL min^{-1}. This typically gave a response of 350−400 response units (RU). Hydroxylase (native or primary amine/arginine modified) at concentrations of 50−300 μg mL^{-1} was passed over the immobilized GST-protein B to assess the level of binding between the hydroxylase and protein B. The flow rate was 5 μL min^{-1} for qualitative detection of binding and 30 μL min^{-1} for quantitative determination of dissociation constants. During each experiment, the hydroxylase sample was also injected through a separate control flow cell with no anti-GST antibody or GST-protein B bound to the chip, to correct for nonspecific binding between the hydroxylase and the chip surface. As an additional control, it was confirmed that no binding was apparent between bovine serum albumin (100 μg mL^{-1}) and the chip activated with anti-GST antibody and GST-protein B. Data analysis for determination of the dissociation constant of the hydroxylase−protein B complex was performed using BIAevaluation software (BIAcore), presuming a hydroxylase molecular mass of 251 kDa.

Table 1: Effect of Cross-Linking and Covalent Modification Reagents on Activity of the Hydroxylase

reagent	assay	specific activity (nmol min^{-1} [mg of hydroxylase]$^{-1}$)
none	whole complex	220 ± 8
	peroxide shunt	98 ± 2
BS^3 (cross-linker)	whole complex	0
	peroxide shunt	97 ± 4
sulfo-NHS-acetate	whole complex	0
(primary amine neutralizer)	peroxide shunt	93 ± 5
p-hydroyphenylglyoxal	whole-complex	0
(arginine modifier)	peroxide shunt	97 ± 4

Mass Spectrometry. The modified and native samples of the hydroxylase were resolved into their constituent subunits by reverse-phase liquid chromatography using a C18 PepMap column (LC Packings) in a Micromass Modular CapLC system (Micromass UK) with a 10−40% acetonitrile gradient in 0.1% formic acid. Electrospray mass spectrometry was performed on-line using a Micromass Q-Tof Ultima API tandem mass spectrometer (Micromass UK).

RESULTS

Reaction of the Hydroxylase with a Cross-Linking Agent Inhibited the sMMO Whole-Complex Reaction but not the Peroxide Shunt. To investigate the significance of the global shape change that is observed in the hydroxylase upon binding to proteins B and the reductase, we cross-linked the hydroxylase dimer $(\alpha\beta\gamma)_2$ with the bivalent amine-specific cross-linking reagent BS^3, in an attempt to prevent relative movement of the two $\alpha\beta\gamma$ protomers of the hydroxylase. This resulted in complete inhibition of propene oxygenation activity when assayed in the whole sMMO complex, consistent with our initial hypothesis that cross-linking would prevent a catalytically essential conformational change. Even more interestingly, cross-linking of the hydroxylase had no effect upon its activity via the peroxide shunt (Table 1), which our previous results had suggested may be independent of the conformational change that we were trying to block (*27*).

Surprisingly, however, SDS−PAGE analysis indicated that only partial cross-linking of the hydroxylase had been obtained since approximately 30% of the α and β subunits had not been cross-linked (compare Figure 1A, lanes 1 and 2). Therefore, the observed complete inhibition of the whole-complex sMMO activity could not be due to the cross-linking alone. During nondenaturing PAGE analysis, the cross-linked hydroxylase (Figure 1B, lane 2) migrated further than the native hydroxylase (Figure 1B, lane 1), presumably due to neutralization of positive charges on primary amine groups of the lysine side-chains and N-termini. Furthermore, only one band was observed for the cross-linked and the native hydroxylase, suggesting that a single charged protein species was present in both cases. In the case of the cross-linked hydroxylase, this also suggested that 100% of the accessible

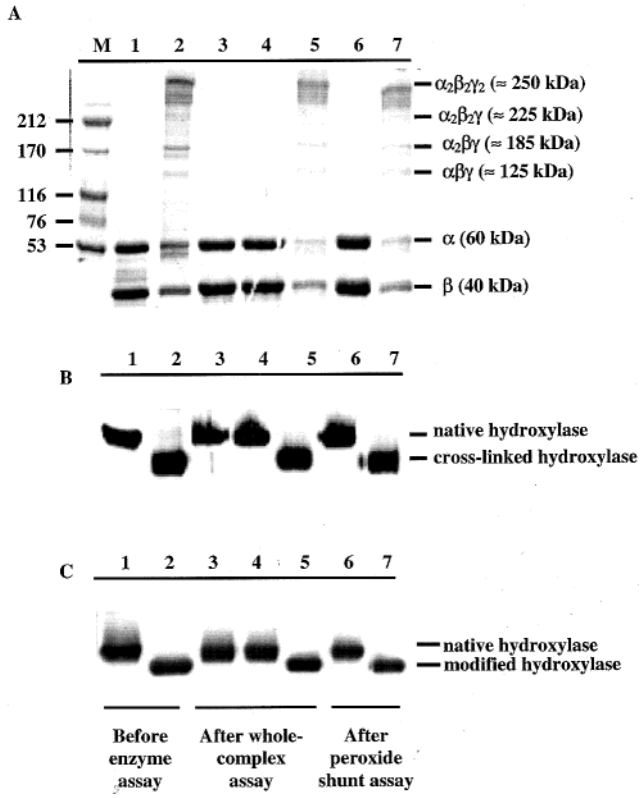

FIGURE 1: Cross-linking and neutralization of primary amino groups associated with the hydroxylase. The hydroxylase was reacted with the cross-linker (BS^3) or the lysine-neutralizing reagent (sulfo-NHS-acetate), and excess reagent was removed by ultrafiltration. Samples from the cross-linking experiment were analyzed by means of (A) SDS−7.5% PAGE and (B) nondenaturing, native PAGE. Samples from the lysine neutralization experiment were analyzed by using nondenaturing PAGE (C). Samples analyzed before measurement of propene oxygenation activity: lanes 1 and 2, unreacted and reacted hydroxylase, respectively. Samples analyzed after measurement of propene oxygenation activity in the sMMO complex: lane 3, unreacted hydroxylase; lane 4, control in which the probe was removed by ultrafiltration before addition of the hydroxylase; lane 5, reacted hydroxylase. Samples analyzed after measurement of propene oxygenation activity via the peroxide shunt: lanes 6 and 7, unreacted and reacted hydroxylase, respectively. The proteins were visualized with Coomassie blue; molecular masses of standards in lane M of part A are indicated in kDa. The cross-linked species observed by means of SDS−PAGE (A) have been tentatively assigned based on the known molecular masses of the α, *β*, and *γ* subunits of the hydroxylase.

primary amine groups had reacted under these conditions. If only partial reaction with the accessible primary amine groups had been obtained at least two bands would have been expected during nondenaturing PAGE owing to different charge states from different extents of reaction with the cross-linker.

The possibility that inhibition of the whole-complex sMMO activity was caused by unreacted cross-linker reacting with the other components of the complex was eliminated by a control experiment. Here the hydroxylase was not added to the reaction until after the cross-linking reagent had been removed by the ultrafiltration procedure. No inhibition of the sMMO activity was observed and no reaction of lysine residues with residual cross-linker was detected by SDS or nondenaturing PAGE (Figure 1A and B, compare lanes 3 and 4). Thus, the inhibition of the whole-complex activity was solely due to the action of the cross-linker on the hydroxylase.

It was also possible that the different effects of the cross-linking reagent on the whole-complex and the peroxide shunt reactions were due to different stabilities of the blocked amine groups under the different conditions of the two enzyme assays. This possibility was eliminated by analyzing samples after each assay, when the amine neutralization/cross-linking was found to be unchanged (Figure 1A and B, lanes 3 and 5−7). Thus, the peroxide shunt reaction was indeed insensitive to modification of the hydroxylase by the cross-linker. Moreover, the effect of the cross-linker upon catalysis by the whole sMMO complex appeared to be due to neutralization of primary amine groups within the hydroxylase and not due to cross-linking of the hydroxylase.

Primary Amine-Specific Modification of the Hydroxylase Affected sMMO Activity in the Same Way as Cross-Linking. To test the hypothesis that it was covalent modification of primary amine groups on the hydroxylase that inactivated the whole-complex reaction, the above experiments were repeated with a monodentate reagent (sulfo-NHS-acetate), which had the same reactive group as the cross-linker but could not form cross-links. As predicted, complete inhibition of the propene oxygenation activity of the sMMO complex was observed but activity via the peroxide shunt was unaffected (Table 1). As observed with the cross-linking reagent, we also detected only a single protein band on the nondenaturing gel, indicating again that 100% of the accessible lysine residues had reacted (Figure 1C). Similar controls to those performed during the cross-linking experiment also confirmed that no excess sulfo-NHS-acetate reacted with protein B or the reductase and that neutralization of the primary amine groups was stable during all enzyme assays (Figure 1C, lanes 3−7). Thus, we proved conclusively that accessible, presumably positively charged, primary amine groups on the hydroxylase were important for the activity of the sMMO complex, but were not required for substrate oxygenation by the hydroxylase alone via the peroxide shunt.

Arginine Modification of the Hydroxylase also Inhibited the sMMO Whole-Complex Reaction. We were interested to see whether the inhibitory effect was a specific one due to neutralization of primary amine groups or a general one due to the modification of positively charged species on the surface of the protein. Inspection of the structure of the hydroxylase (*9*) showed that, at the assay pH of 7.0, most of the positive charges on the surface of the hydroxylase that were not due to primary amines would emanate from arginyl side-chain guanidinium groups. To investigate the importance of these, we used the arginine-specific modifying reagent *p*-hydroxyphenylglyoxal. The results were indistinguishable from those observed with the primary amine-specific agents, i.e., modification of arginyl residues abolished propene epoxygenation activity in the sMMO whole complex reaction but had no effect on the activity via the peroxide shunt (Table 1). Again, control experiments showed that the blocked hydroxylase samples contained no residual blocking reagent, and so the effect of the *p*-hydroxyphenylglyoxal was entirely due to its effect on the hydroxylase. Thus, the sMMO whole-complex reaction, but not the peroxide shunt, was sensitive to inhibition by covalent modification of either of two types of positively charged groups on the hydroxylase. This posed the question, "What is the catalytic function of these positively charged groups?"

Table 2: Effect of Covalent Modification of the Hydroxylase on NADH Oxidation Activity

assay components	rate of NADH oxidation (nmol min^{-1} [mg of hydroxylase]$^{-1}$)
hydroxylase	0
reductase	1.93
hydroxylase + reductase	24.1
hydroxylase + reductase + protein B	20.3
hydroxylase +reductase + protein B + propene	29.4
primary amine-blocked hydroxylase + reductase	1.44
primary amine-blocked hydroxylase + reductase + protein B	2.41
primary amine-blocked hydroxylase + reductase + protein B + propene	1.76
arginine-blocked hydroxylase + reductase + protein B	6.59

Modification of Positively Charged Groups on the Hydroxylase Interfered with Electron Transfer from the Reductase. Covalent modification of positively charged groups associated with the hydroxylase inhibited the sMMO whole-complex reaction, but had no effect on the peroxide shunt reaction, which is catalyzed by the hydroxylase alone. Therefore, it seemed likely that the inhibition of the whole-complex reaction was exclusively due to effects on interactions between the hydroxylase and the reductase and/or protein B and not on the ability of the hydroxylase to interact with its hydrocarbon substrate. The principal chemical requirement of protein−protein interactions in the whole-complex reaction is for delivery of electrons from NADH to the hydroxylase via the reductase. We therefore wished to determine whether the specific inhibition of the whole-complex reaction by covalent modification of the hydroxylase was due to inhibition of such electron transfer.

The flow of electrons from the reductase to the hydroxylase was monitored by measuring NADH oxidase activity (Table 2). As previously observed (*13*), the endogenous NADH oxidase activity of the reductase was low but was substantially increased in the presence of the native hydroxylase. Under these conditions, NADH is oxidized by the reductase, and electrons pass to dioxygen via the hydroxylase in a reaction that is not coupled to substrate oxygenation (*13*). Addition of protein B to the hydroxylase + reductase system slightly suppressed NADH oxidase activity, showing that protein B inhibited uncoupled electron transfer. The 16% suppression of uncoupled electron transfer by protein B (Table 2) was significantly less than the 86% that we observed previously (*13*). A recent report (*18*) actually found stimulation of electron transfer by protein B in the absence of substrate, and so the effect of protein B on electron transfer may be sensitive to the reaction conditions. During the current study, as observed previously (*13*), electron transfer was stimulated by addition of the substrate propene. Under these conditions, NADH oxidation by the reductase is fully coupled to substrate oxygenation at the hydroxylase (*13*). When, however, the native hydroxylase was replaced by the primary amine-blocked form, the increase in NADH oxidase activity by the hydroxylase was effectively abolished, whether protein B and propene were present or not (Table 2). Thus, covalent modification of accessible primary amine groups on the hydroxylase inhibited both coupled and uncoupled transfer of electrons from the reductase to the hydroxylase. Blocking of arginyl side-chains on the hydroxylase severely diminished but did not completely prevent electron transfer (Table 2).

Table 3: Effect of Covalent Modification of the Hydroxylase on Inhibition of the Peroxide Shunt Reaction by Protein B[a]

	specific activity via the peroxide shunt (nmol min^{-1} [mg of hydroxylase]$^{-1}$)		
protein B[a]	native hydroxylase	primary-amine neutralized hydroxylase	arginine modified hydroxylase
0	98 ± 2	93 ± 5	97 ± 4
5	30 ± 3	91 ± 7	93 ± 4

[a] Specific activity was measured at 1 mg mL^{-1} of hydroxylase and expressed in nmol of epoxypropane formed min^{-1} (mg of hydroxylase)$^{-1}$.

Modification of Positively Charged Groups on the Hydroxylase Prevented its Interaction with Protein B. Protein B inhibits the peroxide shunt catalyzed by the native hydroxylase (*28*), and so the peroxide shunt reaction could be used to assess the effect of modification of the hydroxylase on its interaction with protein B. Primary amine group neutralization by sulfo-NHS-acetate and arginine modification by *p*-hydroxyphenylglyoxal prevented the inhibitory effect of protein B during the peroxide shunt reaction (Table 3). Thus, blocking the accessible primary amine groups and arginine side-chains on the hydroxylase clearly interfered with its interaction with protein B under these conditions.

The binding between protein B and the various forms of the hydroxylase was also measured directly by means of surface plasmon resonance (SPR). The GST-protein B fusion protein, which showed full activity when assayed in the presence of hydroxylase and reductase, was immobilized onto a BIAcore CM5 sensor chip via anti-GST monoclonal antibodies. Native and covalently modified hydroxylase samples were flowed across the chip to measure their interaction with the immobilized GST-protein B. As can be seen in Figure 2A, the association of the hydroxylase to immobilized GST-protein B was indicated by an increase in the SPR response after addition of the hydroxylase, followed by a decrease in the SPR response after removal of the hydroxylase, signifying its dissociation from the GST-protein B on the chip. The dissociation constant for the protein B−native hydroxylase complex derived from the SPR data was 0.16 μM, which was in good agreement with the 0.3 μM measured by isothermal titration calorimetry and 0.19 μM deduced from tryptophan fluorescence data (*18*). When, however, primary amine- or arginine-modified hydroxylase was then used with the same GST-protein B-activated chip, the maximum increase in SPR response was diminished by at least 10-fold relative to that seen with native hydroxylase, indicating that the interaction between the proteins was severely inhibited (Figure 2B and C). The association of the primary amine- and arginine-modified hydroxylases with the immobilized protein B was so weak that dissociation constants could not be accurately determined using the range of modified hydroxylase concentrations available (<1.2 μM). The dissociation constants in these cases are estimated to be least several micromolar.

Covalent Modification of the Hydroxylase Occurred at Multiple Sites and Gave Rise to Heterogeneous Samples. The

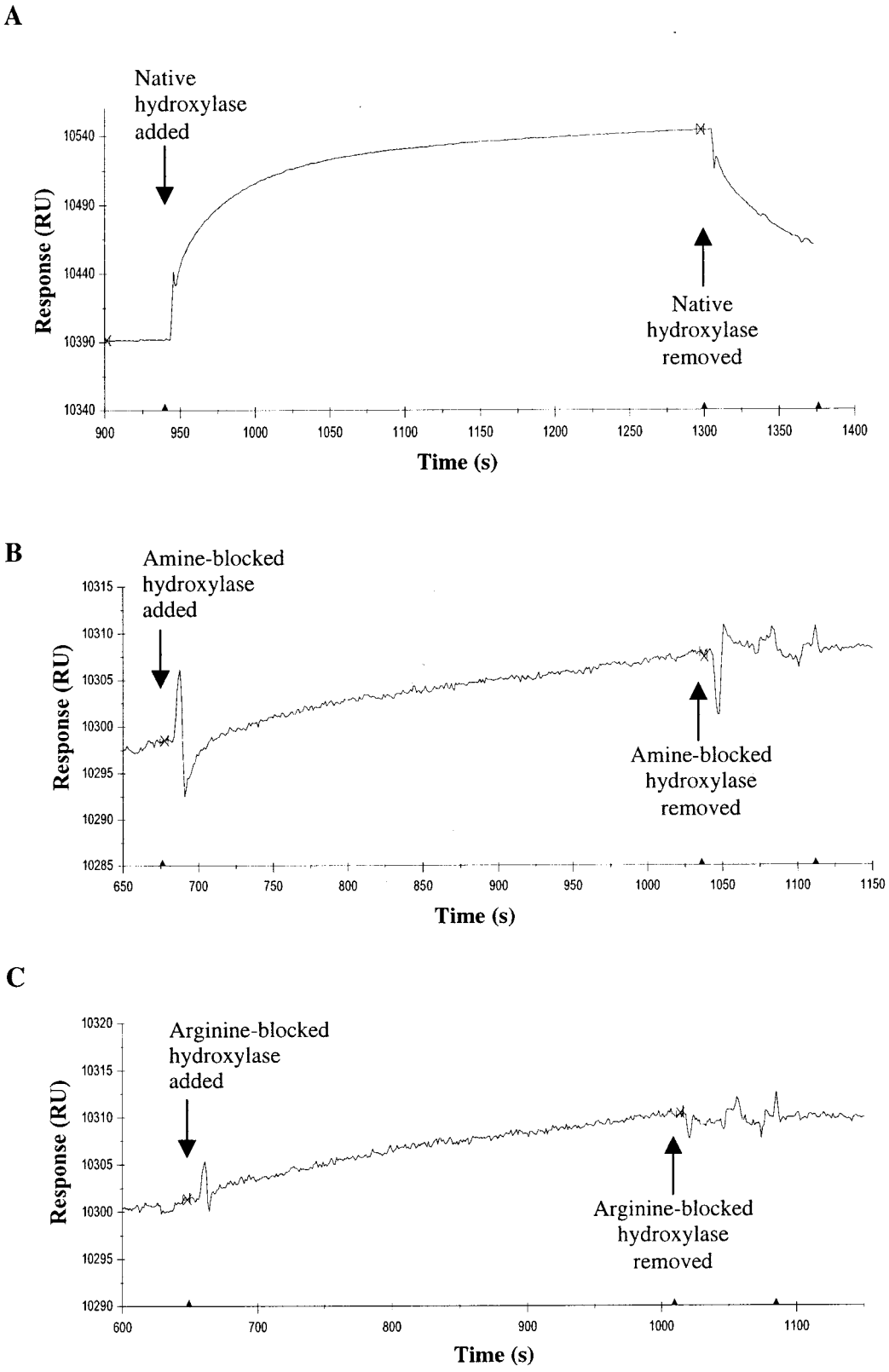

FIGURE 2: Surface plasmon resonance analysis of the binding between the GST-protein B fusion protein and (A) native, (B) primary amine-modified, and (C) arginine-modified hydroxylase. The points at which the hydroxylase was added to and removed from the buffer flowing across the sensor chip are indicated.

extent of reaction of the hydroxylase with the modification reagents was assessed by analysis of modified hydroxylase samples after removal of residual modification reagent by repeated ultrafiltration and dilution. As an additional control, the activity of the peroxide shunt reaction and the inactivity via the whole-complex assay were confirmed for each modified hydroxylase sample before quantitation of the modifying reagent. The increase in A_{340} after reaction with *p*-hydroxyphenylglyoxal hydroxylase suggested that an average of 2.3 mol of the arginine-specific reagent had reacted

per mol of hydroxylase dimer $(\alpha\beta\gamma)_2$, if a molar extinction coefficient of 18 300 M^{-1} cm^{-1} is presumed for the protein-associated modification reagent (*37*).

In contrast to the low-resolution results obtained by using nondenaturing PAGE (Figure 1C), mass spectrometry of samples of native and modified hydroxylase revealed that the blocking patterns with both reagents were complex and heterogeneous. The ranges of masses observed for the β and γ subunits of the primary amine-modified hydroxylase could be accounted for by addition of between 4 and 21 acetyl groups to the β subunit and 1−4 such groups to the γ subunit. The β subunit of the arginine-modified hydroxylase produced molecular ions with masses corresponding to the native subunit plus 0, 1, 2, or 3 additions of 130−133 Da. This was consistent with the 132 Da that would be expected per addition of one molecule of *p*-hydroxyphenylglyoxal (*38*). The arginine-specific reagent did not significantly change the mass spectrum of the γ subunit, suggesting that no arginyl residues on this component were modified. The mass spectra of both the primary amine- and arginine-modified α subunits were very complex, probably due to heterogeneity of modification combined with the fragmentation of the molecular ions that was observed with the native α subunit. Consequently, deconvolution of the spectra and assignment of masses were impossible. While these results confirmed that the α subunits were modified, they gave no reliable information about the number of positive charges affected.

Primary Amine and Arginine Groups on Protein B Are Not Required for Interaction with the Hydroxylase. The roles of accessible positively charged residues on protein B were also investigated. Neither primary amine- nor arginine-blocking of protein B had any measurable effect on its activity in the whole sMMO complex reaction nor its inhibitory effect on the peroxide shunt reaction (data not shown). These results were consistent with a model in which a catalytically productive interaction between protein B and the hydroxylase requires electrostatic forces between positively charged groups on the hydroxylase and negatively charged groups on protein B.

DISCUSSION

During this study, the use of an amine cross-linking reagent to investigate a conformational change induced by protein−protein interactions in the sMMO complex has revealed a previously unknown role for accessible positively charged groups on the sMMO hydroxylase. Primary amino groups (i.e., lysyl side-chains and/or amino termini) and arginine side-chain guanidinium ions have been shown to be required for binding between the hydroxylase and protein B and to be essential for functional interactions that the hydroxylase forms with protein B and the reductase.

Importance of Ionic Interactions in the sMMO Complex. Of the catalytic properties investigated, only electron transfer from the reductase was affected to significantly different extents by primary amine- and arginine-specific modification of the hydroxylase. Throughout the rest of the study, modification of primary amino and arginyl groups had almost identical effects. Therefore, if the modification reagents showed their established specificities in these experiments, it appears either that interaction of the components of sMMO has a general requirement for positive charges on the hydroxylase or that at least one positively charged moiety of each type is involved in each of the processes investigated. The primary amine-modifying reagent, sulfo-NHS-acetate, may exert its effect simply by neutralizing the positive charges on the amino groups, since the acetylated amino groups would be uncharged at neutral pH. The effect of arginine modification by *p*-hydroxyphenylglyoxal are probably, however, at least partly steric, because both of the heterocyclic derivatives produced by reaction of arginine with the chemically similar modification reagent phenylglyoxal are positively charged (*38*). Indeed, modification of primary amine groups or arginine side-chains may destabilize protein−protein interactions by introducing new steric bulk at the interfaces where the molecules interact. Previous experimental evidence for the stabilization of the protein B-hydroxylase interaction by 1 M NaCl (*26*) suggested involvement of (salt-stabilized) hydrophobic interactions rather than (salt-destabilized) electrostatic forces. In light of the current study, it is clear that both types of interaction are important.

The requirement for positive charges on the hydroxylase for interaction with protein B implies that complementary negative charges on protein B are also needed. Previously, when EDC was used to cross-link the sMMO of *Ms. trichosporium* OB3b, brief exposure to EDC inactivated the sMMO components without cross-linking, and protein B was more sensitive than the hydroxylase to this form of inactivation (*20*). These results may be explained if it is assumed, as previously suggested (*20*), that inactivation was due to modification of accessible carboxylate groups by EDC. Thus, if carboxylate groups on protein B are required to interact with the positive charges on the hydroxylase, protein B may be more sensitive than the hydroxylase to a reagent that modifies primarily carboxylates. A recent nuclear magnetic resonance study, also using the sMMO from *Ms. trichosporium* OB3b, has given further evidence for the role of carboxylate groups on protein B. Addition of the hydroxylase to protein B caused negatively charged residues on protein B to be perturbed more than protein B-associated positively charged moieties, suggesting that the negatively charged groups on protein B are closely involved in interactions with the hydroxylase (*39*).

Mechanistic Implications. Inhibition of electron transfer from the reductase to the hydroxylase by covalent modification of the hydroxylase was observed directly in the NADH oxidase assays. These results are consistent with the inactivity of covalently modified hydroxylase in the whole-complex reaction (which requires electron transfer from NADH via the reductase) and its activity in the peroxide shunt (which does not require electrons from NADH). Positively charged groups on the hydroxylase must therefore be (i) essential for binding of the reductase to the hydroxylase, and/or (ii) required for a reductase-induced conformational change that is needed for electron transfer, and/or (iii) directly involved in the electron-transfer pathway.

The abolition of whole-complex sMMO activity by covalent primary-amine modification of the hydroxylase could be explained completely by the observed effect on electron transfer from the reductase. However, electron transfer was not completely inhibited by arginine modification, and so the complete loss of the whole-complex activity seen with arginine-modified hydroxylase must arise at least

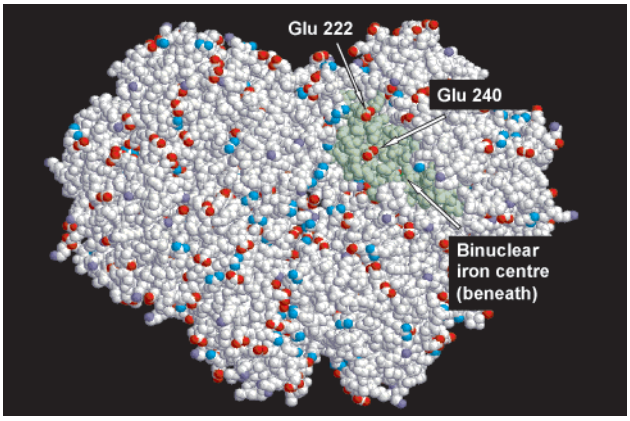

FIGURE 3: Distribution of charged groups on the surface of the hydroxylase, based on the X-ray crystal structure (*9*). Nitrogen atoms of primary amino and arginyl side-chain guanidinium groups are shown in dark and light blue, respectively. Side-chain carboxylate oxygen atoms are shown in red. The uncharged parts of helices αE and αF of the α subunit, proposed to form the hydrophobic binding site for protein B, are colored light green.

partly from another source, e.g., loss of productive interaction with protein B. Consistent with this, covalent modification of the hydroxylase inhibited binding of protein B and prevented the inhibitory effect of protein B during the peroxide shunt reaction. Protein B did, however, cause a significant increase in NADH oxidation in the primary amine-modified hydroxylase + reductase systems (Table 2), and so protein B may be able to stimulate a limited amount of electron transfer from the reductase to the primary amine-modified hydroxylase.

Structural Implications. The distribution of primary amine and arginyl side-chains on the surface of the hydroxylase is remarkably uniform (Figure 3). It is therefore difficult to speculate confidently about which positively charged groups may be important in the absence of direct evidence to identify the sites of protein−protein interaction. The mass spectrometric data obtained for the primary amine-blocked hydroxylase were complex and showed heterogeneous modification of all three subunits, thus offering little information about the primary amine groups that are crucial for intercomponent interactions. Analysis of the arginine-modified hydroxylase, however, showed that a substantial amount of the β and γ subunits remained unmodified even though the activity of the hydroxylase in the whole-complex assay was completely inhibited. This strongly suggests that the critical arginyl residue(s) required for intercomponent interactions and/or proton/electron transfer are located on the α subunit. Moreover, the observation that on average only 2.3 molecules of *p*-hydroxyphenylglyoxal reacted with each molecule of hydroxylase dimer suggests that the observed effects of arginine modification may result from derivatization of a single amino acid on each α subunit. Identification of such an essential arginyl residue may provide experimental evidence for the site on the hydroxylase via which it interacts with the reductase or protein B.

One possible binding site for protein B, suggested by modeling studies (*25*), is formed by a predominantly hydrophobic cleft in the α subunit of the hydroxylase, near to its interface with the β subunit. This cleft is lined by the protruding faces of helices αE and αF, which form part of the four-helix bundle that coordinates the binuclear iron active center (*25*, *40*). It is devoid of positive charges, and so, if protein B does bind here, the positive charges important in its binding must be among the lysyl and arginyl side-chains on the surrounding protein surface (Figure 3). The cleft contains two carboxylate groups (belonging to Glu 222 and Glu 240; Figure 3), which suggest ionic interactions to positive charges on protein B. Since modification of primary amines and arginines on protein B did not affect catalysis, such presumably essential positive charges on protein B could arise from its two histidine residues (His 33 and His 140). Alternatively, sterically hindered but positively charged modified arginyl side-chain(s) on the arginine-blocked protein B may still be able to form productive ionic interactions with the carboxylate groups in the cleft on the hydroxylase, or the binding site for protein B may lie elsewhere.

CONCLUSIONS

The results presented here show that covalent modification of the sMMO hydroxylase allows functional separation of the processes required for substrate oxygenation from those involved in delivery of electrons into the active site and modulation of sMMO activity by protein B. These results lend credence to a model of sMMO in which protein B and/or the reductase induce a conformational change in the hydroxylase that is necessary for delivery of electrons into the binuclear iron site but not for substrate oxygenation per se. We propose that covalent modification of positively charged residues on the hydroxylase prevents this conformational change by interfering with the intermolecular interactions that bring it about. In addition, it is possible that one or more accessible positively charged residues on the hydroxylase may be directly involved in the electron or proton relay pathway. Specific chemical and mutational modification of the hydroxylase, to obtain direct experimental evidence for the sites of interaction of the components of sMMO, the pathway of electron transfer, and the role of conformational changes in catalysis, is the subject of our continuing research.

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REFERENCES

1. Hanson, R. S., and Hanson, T. E. (1996) *Microbiol. Rev. 60*, 439−471.
2. Dalton, H. (1991) in *Applications of Enzyme Biotechnology* (Kelly, J., Ed.) pp 55−68, Plenum Press, New York.
3. Stanley, S. H., Prior, S. D., Leak, D. J., and Dalton, H. (1983) *Biotechnol. Lett. 5*, 487−492.
4. Colby, J., and Dalton, H. (1976) *Biochem. J. 157*, 495−497.
5. Colby, J., Stirling, D. I., and Dalton, H. (1977) *Biochem. J. 165*, 395−402.
6. Green, J., and Dalton, H. (1989) *J. Biol. Chem. 264*, 17698−17703.
7. Colby, J., and Dalton, H. (1978) *Biochem. J. 171*, 461−468.

8. Dalton, H., Wilkins, P. C., and Jiang, Y. (1993) in *Microbial Growth on C_1 Compounds* (Murrell, J. C., and Kelly, D. P., Eds.) pp 65−80, Intercept, Andover, UK.
9. Rosenzweig, A. C., Frederick, C. A., Lippard, S. J., and Nordlund, P. (1993) *Nature 366*, 537−543.
10. Rosenzweig, A. C., Nordlund, P., Takahara, P. M., Frederick, C. A., and Lippard, S. J. (1995) *Chem. Biol. 2*, 409−418.
11. Ericson, A., Hedman, B., Hodgson, K. O., Green, J., and Dalton, H. (1988) *J. Am. Chem. Soc. 110*, 2330−2332.
12. Woodland, M. P., Patil, D. S., Cammack, R., and Dalton, H. (1986) *Biochim. Biophys. Acta 873*, 237−242.
13. Lund, J., Woodland, M. P., and Dalton, H. (1985) *Eur. J. Biochem. 147*, 297−305.
14. Lund, J., and Dalton, H. (1985) *Eur. J. Biochem. 147*, 291−296.
15. Liu, K. E., and Lippard, S. J. (1991) *J. Biol. Chem. 266*, 12836−12839.
16. Kazlauskaite, J., Hill, H. A. O., Wilkins, P. C., and Dalton, H. (1996) *Eur. J. Biochem. 241*, 552−556.
17. Froland, W. A., Andersson, K. K., Lee, S. K., Liu, Y., and Lipscomb, J. D. (1992) *J. Biol. Chem. 267*, 17588−17597.
18. Gassner, G. T., and Lippard, S. J. (1999) *Biochemistry 38*, 12768−12785.
19. Liu, K. E., Valentine, A. M., Wang, D. L., Huynh, B. H., Edmondson, D. E., Salifoglou, A., and Lippard, S. J. (1995) *J. Am. Chem. Soc. 117*, 10174−10185.
20. Fox, B. G., Liu, Y., Dege, J. E., and Lipscomb, J. D. (1991) *J. Biol. Chem. 266*, 540−550..
21. Lipscomb, J. D. (1994) *Annu. Rev. Microbiol. 48*, 371−399.
22. Davydov, R., Valentine, A. M., Komar-Panicucci, S., Hoffman, B. M., and Lippard, S. J. (1999) *Biochemistry 38*, 4188−4197.
23. Paulsen, K. E., Liu, Y., Fox, B. G., Lipscomb, J. D., Munck, E., and Stankovich, M. T. (1994) *Biochemistry 33*, 713−722.
24. Pulver, S. C., Froland, W. A., Lipscomb, J. D., and Solomon, E. I. (1997) *J. Am. Chem. Soc. 119*, 387−395.
25. Walters, K. J., Gassner, G. T., Lippard, S. J., and Wagner, G. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 7877−7882.
26. Brandstetter, H., Whittington, D. A., Lippard, S. J., and Frederick, C. A. (1999) *Chem. Biol. 6*, 441−449.
27. Gallagher, S. C., Callaghan, A. J., Zhao, J., Dalton, H., and Trewhella, J. (1999) *Biochemistry 38*, 6752−6760.
28. Jiang, Y., Wilkins, P. C., and Dalton, H. (1993) *Biochim. Biophys. Acta 1163*, 105−112.
29. Haugen, D. A., and Coon, M. J. (1976) *J. Biol. Chem. 251*, 7929−7939.
30. Hoeven, T. A. V. D., Haugen, D. A., and Coon, M. J. (1974) *Biochem. Biophys. Res. Commun. 60*, 569−575.
31. White, R. E., and Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315−356.
32. Pilkington, S. J., and Dalton, H. (1990) *Methods Enzymol. 188*, 181−190.
33. Lloyd, J. S., Bhambra, A., Murrell, J. C., and Dalton, H. (1997) *Eur. J. Biochem. 248*, 72−79.
34. Whitaker, J. R., and Granum, P. E. (1980) *Anal. Biochem. 109*, 156−159.
35. Scopes, R. K. (1974) *Anal. Biochem. 59*, 277−282.
36. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
37. Yamasaki, R. B., Vega, A., and Feeney, R. E. (1980) *Anal. Biochem. 109*, 32−40.
38. Krell, T., Pitt, A. R., and Coggins, J. R. (1995) *FEBS Lett. 360*, 93−96.
39. Chang, S.-L., Wallar, B. J., Lipscomb, J. D., and Mayo, K. H. (2001) *Biochemistry 40*, 9539−9551.
40. Rosenzweig, A. C., Brandstetter, H., Whittington, D. A., Nordlund, P., Lippard, S. J., and Frederick, C. A. (1997) *Proteins 29*, 141−152.

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