Structural Features of Covalently Cross-linked Hydroxylase and Reductase Proteins of Soluble Methane Monoxygenase as Revealed by Mass Spectrometric Analysis

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Methanotrophic bacteria express a membrane-bound enzyme termed particulate methane monoxygenase to convert methane to methanol. When copper is unavailable some methanotrophs, including Methylococcus capsulatus (Bath) and Methylosinus trichosporum OB3b, employ an iron-containing soluble methane monoxygenase (sMMO)1 (1).

The sMMO system comprises several proteins. The αβγδ hydroxylase (MMOH, 251 kDa) contains a glutamate-bridged diiron active site in each α subunit. The crystal structure of MMOH has been determined in several redox states and with various products and substrate analogs bound (2–6). An iron-sulfur flavoprotein reductase (MMOR, 38.5 kDa) transfers electrons from NADH to MMOH. The solution structure of the N-terminal [2Fe-2S] domain of MMOR is available (7). A cofactorless regulatory protein (MMOB, 15.9 kDa) alters the properties of the diiron site and is required for activity, and its solution structure has also been determined (8, 9). A fourth protein (MMOD, component D of sMMO), 11.9 kDa) binds to the hydroxylase and inhibits catalysis in vitro, but its function has yet to be determined (1, 10).

Complex formation between MMOH, MMOR, and MMOB is required for sMMO catalysis. The catalytic cycle begins with the diiron site of MMOH in its Fe(III)/Fe(III) resting state. MMOR then docks to MMOH, transfers two electrons (derived from NADH) to each diiron(III) active site, and reduces MMOH to the Fe(II)/Fe(II) state. In the presence of MMOB, reduced MMOH reacts with O2 to generate a series of intermediates that hydroxylate substrates. An MMOB:MMOH ratio of 2 produces maximal activity. Binding of MMOB also alters the redox potentials and spectroscopic properties of the diiron center in MMOH and accelerates electron transfer from MMOR (1).

Despite the importance of complexes involving these proteins in the catalytic cycle, only limited structural information is available about them. More than 10 years ago (11), it was demonstrated that the proteins of sMMO could be covalently cross-linked by the reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC). SDS-PAGE analysis of the reaction products allowed identification of the polypeptides involved in cross-links. These early studies located some of the proximal polypeptides of the MMOH holoprotein before its crystal structure was available (11, 12).

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§§ The on-line version of this article (available at http://www.jbc.org) contains Table S1.
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Methanotrophic bacteria rely on metalloenzymes to catalyze methane hydroxylation (Equation 1), the first step in the metabolic pathway that supplies all their cellular carbon and energy.

\[
\text{CH}_4 + \text{O}_2 + \text{NADH} + \text{H}^+ \rightarrow \text{CH}_3\text{OH} + \text{H}_2\text{O} + \text{NAD}^+ \quad \text{(Eq. 1)}
\]
Several other methods have been used to study complexes of the sMMO proteins. NMR titrations indicated specific residues on MMOB and MMOR-Fd that interact with MMOH (7, 8). Small-angle x-ray scattering studies led to a model of a ternary MMOH-MMOB-MMOR complex in which MMOH undergoes a large structural rearrangement (14). Modification of positively charged residues on the surface of MMOH inhibits the binding of MMOB and electron transfer from MMOR (15). Measurements of the distance between the hydroxylase diiron site and a site-directed spin label on MMOB provide information about where MMOB may contact MMOH (16).

In the present study we have applied mass spectrometry to identify specific amino acid residues that are cross-linked by EDC in complexes of the sMMO proteins. Of particular interest were those cross-links between MMOH, where the catalytic non-heme diiron center is located, and the other protein components. The results allow definitive statements to be made concerning the interaction of the ferredoxin domain of MMOR with MMOH and pave the way to future experiments that apply this methodology.

EXPERIMENTAL PROCEDURES

Reagents and General Techniques—The EDC cross-linking reagent was purchased from Pierce, and other biochemicals were from Sigma. Solvents used in LC runs were purchased from Burdick and Jackson (Muskegon, MI) and were of high performance liquid chromatography grade or higher.

Protein Purification—MMOH was isolated from cultures of M. capsulatus (Bath), as previously described (17). Consistent with previous work, purified MMOH contained 3.9–4.0 mol of iron/mol of protein, and specific activity for conversion of propylene to propylene oxide at 45 °C was in the range 200–300 nmol min
–1 mg
–1 MMOH. Conditions for purification of recombinant MMOR, MMOR-FAD, and MMOR-Fd from Escherichia coli are reported elsewhere (18, 19).

EDC Cross-linking Reactions—Typically, solutions of 10–20 μg protein in 25 mM MOPS, pH 7.0, were allowed to react with 25 or 50 mM EDC for 5 min at ambient temperature. Reactions were quenched by the addition of an equal volume of 2× SDS loading buffer containing 200 mM dithiothreitol.

In-gel Proteolytic Digestion—Aliquots of the products from the cross-linking reactions were separated by SDS-PAGE using either 7.5 or 4–20% Ready Gels (Bio-Rad). Gels were stained either with Coomassie Blue or with zinc. Zinc staining was carried out by soaking the gel in 0.2 M ZnSO4. When the desired degree of opacity was reached, the gel was fixed in 25 mM MOPS, pH 7.0, were allowed to react with 25 or 50 mM EDC, and finally, 1 extraction with 100 mM dithiothreitol.

MALDI Matrix was 2,5-dihydroxybenzoic acid, and the MALDI matrix was in the range 200–200 mM dithiothreitol.

A site-directed spin label on MMOB provide information about where MMOB and electron transfer from MMOR (15). Measure-
tions of MMOB and MMOR-Fd that interact with MMOH (7, 8, 13).

In-gel Proteolytic Digestion—Alcohols of the products from the cross-linking reactions were separated by SDS-PAGE using either 7.5 or 4–20% Ready Gels (Bio-Rad). Gels were stained either with Coomassie Blue or with zinc. Zinc staining was carried out by soaking the gel in 0.2 M imidazole for 10 min, then rinsing briefly with water and incubating in 0.2 M Na2S2O4. When the desired degree of opacity was reached, the gel was treated with 30 μl of 100 mM iodoacetamide in 100 mM acetate buffer, pH 5.0, 50% acetonitrile, then dehydrated with 100 μl acetonitrile and dried in a centrifugal evaporator.

Gel pieces were treated with 30 μl of 20 mM dithiothreitol in 100 mM ammonium bicarbonate and 5% acetonitrile and incubated for 1 h at 55 °C. Dithiothreitol was removed by washing with 100 μl of 100 mM ammonium bicarbonate, then with 100 μl of acetonitrile. Cysteines were alkylated by adding 30 μl of 100 mM iodoacetamide in 100 mM ammonium bicarbonate and incubating for 30 min at room temperature in the dark. Gel pieces were washed twice with 100 mM ammonium bicarbonate and acetonitrile before drying in a centrifugal evaporator.

Gel pieces were rehydrated with a small volume of digestion solution (50 mM ammonium bicarbonate, pH 8.5, and sequencing grade trypsin (Sigma or Promega, Madison, WI). Trypsin was used at an enzyme:substrate ratio of ~1:100 by weight. Digestion was allowed to proceed overnight at 37 °C.

Peptides were extracted from the gel pieces with 100 μl of 20 mM ammonium bicarbonate followed by 2 extractions of 100 μl of 1:1 water: acetonitrile plus 1% trifluoroacetic acid and, finally, 1 extraction with 100 μl of acetonitrile. All extracts were combined in a fresh tube, flash-frozen, and dried in a centrifugal evaporator. Dried extracts were stored at −20 °C until analyzed.

Mass Spectrometry—MALDI-TOF MS. Extracted peptides were analyzed using both the Finnigan MAT Vision 2000 MALDI-TOF (Thermo Finnigan, San Jose, CA) and the Bruker Reflex IV (Bremen, Germany) reflectron mass spectrometers equipped with ultraviolet lasers (nitrogen, 337 nm). The MALDI matrix was 2,5-dihydroxybenzoic acid, and typically 50–900 laser shots were summed for each spectrum. The laser power used was between 33 and 80%.

Capillary LC MS—An LC Packings capillary LC ( Dionex; Cambridge, MA) coupled to an Applied Biosystems Inc. (Foster City, CA) Sciex QSTAR quadrupole orthogonal time-of-flight (QqTOF) mass spectrometer was employed using information-dependent acquisition. Peptide separation was achieved by using a 256-μm internal diameter × 28-cm homemade capillary column packed with Microm (Auburn, CA) Magic C18 as the stationary phase. A 100-min gradient from 98:2 H2O:CH3CN with 0.1% HCOOH (A) and 85:10:5 CH3CN:CH3OHH2O with 0.1% HCOOH (B) going from 5% to 85% B was run at 1 μl/min. Eluent was sprayed at 4500 V, and tandem MS data were generated with collision energies of 16, 24, and 35 eV for each selected peptide. Data from capillary LC runs were standardized by internal calibration. A mass selection window of ~2.5–3 Da, dependent on mass value, was used allowed for isolation of the isotopic cluster.

MS Data Analysis—LC/MS data were analyzed with Q Analyst software (Applied Biosystems Inc., Foster City, CA). Tryptic peptide masses were calculated from amino acid sequences of sMMO proteins based on DNA sequencing results (20) using the programs PEPTIDEMASS (21) or GPMAW (22). Observed masses were manually matched to calculated values to make assignments. Mascot (Matrix Science, Ltd., London, UK; www.matrixscience.com) was also used to analyze tandem MS data (23).

Site-directed Mutagenesis—The MMOR variant carrying E56Q and E91Q mutations was prepared from the plasmid pRED21 according to the QuikChange method (Stratagene, La Jolla, CA). pRED21 contains the M. capsulatus (Bath) mmoC gene in a pET21 vector. The E56Q mutation was introduced first with the primer 5′-GCAAGGCTTGTG-GACGCAAGGGTACACTGGC-3′ and its reverse complement. Positive clones for the E56Q mutation were selected and sequenced at the MIT Biopolymers Laboratory using an ABI 3730 sequencer. The E91Q mutation was introduced in the E56Q background using the primer 5′-CCGAAAGGCCGACTCTGCAATCGAATGCCTATAC-3′ and its reverse complement. The DNA sequence of the double mutant was similarly verified, and the plasmid pRED21 E56Q/E91Q was transformed into E. coli BL21(DE3). Expression and purification were carried out in the same manner as for MMOR (18). MMOR E56Q/E91Q is hereafter designated MMOR EQ2.

NADH Consumption Assays—The activity of sMMO was measured by combining 1 μM MMOH and 2 μM MMOB with varying amounts of MMOR or MMOR EQ2 (17). Propylene-saturated buffer (25 mM MOPS, pH 7.0) was added to give a final propylene concentration of 0.8 mM. Reactions, thermostatted at 25 °C, were initiated by the addition of NADH to a final concentration of 160 μM in a total volume of 400 μl in a quartz cuvette. The absorbance at 340 nm was measured for 2 min, and the rate of NADH consumption was calculated as a linear fit of ΔA340 versus time.

RESULTS

Cross-linking of MMOR-Fd and MMOR to MMOH.—EDC facilitates formation of amide bonds between amine and carboxylic acid groups affording intra- or intermolecular protein cross-links (Fig. 1A). Products resulting from EDC cross-linking of MMOH, polypeptides, an MMOH-MMOR-Fd complex, and an MMOH-MMOR-MMOR complex are revealed in Fig. 1B. MMOH alone forms several intramolecular cross-links, including MMOH-MMOMOHpMOMOH-MMOMOHp, and with extended reaction times, MMOH-MMOMOHp-MMOMOHp where the hyphen denotes cross-link formation between two peptide chains. The band arising from cross-linking between MMOR-Fd and MMOR-Fd was assigned as MMOH-MMOR-Fd based on its mobility and confirmed by proteolytic digestion and mass spectrometry (see below). Full-length MMOR also cross-links to MMOHp, in contrast to previous findings for sMMO from M. trichosporium OB3b (11). The predicted molecular mass of an MMOH-MMOR cross-link, 99.1 kDa, lies between those of MMOH-MMOMOHp (105.6 kDa) and MMOH-MMOMOHp (90 kDa), and the band assigned to the MMOH-MMOR cross-link migrates at that position. If MMOR were cross-linked to MMOH, the resulting band would have a molecular mass of 83.5 kDa.

a J. Muller and S. J. Lippard, unpublished results.
and would migrate more quickly than MMOH$_{\mu}$-MMOH$_{\mu}$ on SDS-PAGE.

Incubation of MMOH and the FAD domain of MMOR (MMOR-FAD) with EDC did not produce a cross-link (data not shown). Thus, the residues of MMOR that cross-link to MMOH lie exclusively within the [2Fe-2S] domain of MMOR. A mixture of MMOR-Fd and MMOR-FAD did not cross-link upon treatment with EDC, suggesting that these domains do not interact strongly in the full-length protein. Electron transfer kinetics and isothermal titration calorimetry experiments support such a conclusion.

Identification of Cross-linked Peptides—The identity and structures of two cross-linked peptides were confirmed by tandem MS analysis of peaks in the MS spectrum, the mass values of which suggested that they might represent cross-linked peptides (Figs. 3 and 4). These results indicated that, in the presence of EDC, the N-terminal amine of MMOH$_{\mu}$ forms amide bonds with the carboxylate side chains of MMOR-Fd Glu-56 and Glu-91 (Figs. 3A and 4A). A peptide with [M + 3H]$^{+}$/z 648.4, elutes at 33.5 min. This mass closely matches that predicted for a cross-link between the tryptic peptides MMOR-Fd 52–62 and MMOH$_{\mu}$ 2–8$^3$ (M$_r$ calc = 1941.93). The tandem mass spectrum recorded after fragmentation of the triply charged form of this peptide (m/z [M + 3H]$^{+}$/z 648.4) is shown in Fig. 3B, and the data are presented in Table I. The entire y-ion series is present, and the assignment of Glu-56 of MMOR-Fd (rather than Asp-58 or Asp-60) as the cross-linking site is unambiguous.

A second cross-link was found that also involves the N-terminal amine of MMOH$_{\nu}$ (Fig. 4A). At 42.5 min, a peptide eluted with $M_r$ obs = 2002.09, matching $M_r$ calc = 2002.03 for a cross-link between MMOR-Fd 88–98 and the MMOH$_{\nu}$ 2–8. MMOR-Fd 88–98 includes three possible sites of cross-linking, Asp-89, Glu-91, and Glu-93, but the tandem mass spectrum unambiguously locates Glu-91 as the only site of cross-link formation (Fig. 4B and Table III).

**FIG. 2.** Total ion chromatogram for an LC/MS run of tryptic peptides of MMOH$_{\nu}$-MMOR-Fd-cross-linked band. Peaks containing cross-linked peptides are indicated by the asterisks.

**TABLE I**

| Protein | No. of unique peptides | Residues observed/total | % coverage |
|---------|------------------------|-------------------------|------------|
| MMOH$_{\nu}$ | 36 | 359/326 | 68 |
| MMOR-Fd | 11 | 90/98 | 92 |
| MMOH$_{\mu}$ | 12 | 126/128 | 32 |

We begin numbering of MMOH$_{\nu}$ from the initial Met even though it is missing from the mature protein as expressed in *M. capsulatus* (Bath) (25). Thus, the N-terminal Ala residue is assigned number 2. We use this numbering scheme to be consistent with our previous studies.
Identity of Cross-link between MMORh and MMOH

The MMOR EQ2 Mutant—To confirm that the EDC-promoted cross-links between MMOHh and MMOR or MMOR-Fd involve the sites identified, the double mutant MMOR E56Q/E91Q (MMOR EQ2) was prepared. MMOR EQ2 was expected to behave similarly to MMOR in most respects but to lack the ability to form the identified cross-links. Indeed, the UV-visible spectrum of purified MMOR EQ2 is identical to that of MMOR. Similar levels of NADH oxidase activity (Table IV) in the two variants indicate that binding of NADH, reduction and re-oxidation of cofactors are not seriously affected by the mutations. Steady-state activity of the sMMO system, however, is impaired with MMOR EQ2.

A comparison of EDC cross-linking of MMOR and MMOR EQ2 to MMOH is made in Fig. 6. MMOR EQ2 still forms cross-links to MMOHh, although the yield of cross-linked material is significantly lower than for MMOR.

DISCUSSION

Mass spectrometric analysis of protein complexes has matured in recent years into a powerful technique (28). A common method of sample preparation, applied here, is to separate a complex by SDS-PAGE, excise a band of interest, and treat it with a protease. The identities of peptides thus generated can be accurately determined because of the high sensitivity, mass accuracy, and sequencing capabilities of modern mass spectrometers. The polypeptide components of the complex can be identified by comparison to a sequence data base. In some cases, mass spectrometry can locate specific sites of chemical cross-linking within a complex. Interaction sites of protein complexes involved in vision (29, 30), DNA replication (31–33), and interprotein electron transfer (34, 35) have all been studied in this manner.

LC/QqTOF MS Analysis of MMOHh-MMOR-Fdh—The identities of two EDC-promoted cross-links between MMOHh and MMOR-Fd involve amide bond formation between the N-terminal amino group of MMOHh and the Glu-56 or Glu-91 side chain of MMOR-Fd. The close proximity of Glu-56 and Glu-91 in the solution structure of MMOR-Fd (Fig. 5) suggests that the two cross-links represent a common interaction between the two proteins. That interaction presumably involves electrostatic attraction between the positively charged N terminus of MMOHh and a negatively charged region containing Glu-56 and Glu-91 of MMOR or MMOR-Fd.

Based on their abundance in the LC/MS data, we conclude...
that the MMOH Ala-2-MMOR-Fd Glu-56/Glu-91 cross-links account for a significant fraction of total cross-linking. Cross-links involving additional residues are also present, however. Fig. 6 reveals that MMOR EQ2, which lacks carboxylates at positions 56 and 91, still forms cross-links to MMOH. The sites of these cross-links are as yet unidentified, but determination of their nature will be facilitated by use of the MMOR EQ2 mutant or the analogous double mutation of MMOR-Fd.

**Implications Regarding the MMOH-MMOR Complex**—Although the occurrence and identity of the cross-links are not in doubt, we must consider two issues before drawing any further conclusions. Does MMOR-Fd faithfully model the interaction of MMOR with MMOH and, if so, does the cross-link reflect a functionally important MMOH-MMOR-Fd complex?

We have several lines of evidence to support the conclusion that MMOR-Fd recapitulates the important features of the MMOH-MMOR complex. Dissociation constants for binding of MMOR and MMOR-Fd to MMOH, measured by isothermal titration calorimetry, are within an order of magnitude of one another (17, 19). Chemically reduced MMOR-Fd transfers electrons efficiently to the diiron center of MMOH (19). Finally, MMOR-FAD does not cross-link to MMOH, suggesting that it interacts weakly if at all with MMOH (data not shown).

Among MMOR proteins from several species, only Glu or Asp residues occur at position 56, and only Gln or His occur at position 91 (7). MMOH protein sequences are highly conserved along the entire polypeptide, including the N terminus (20). Mutation of Glu-56 and Glu-91, both to Gln, results in reduced activity of the sMMO system. It is possible that the cross-link does not reveal a functionally important protein complex but, rather, reflects the greater reactivity of these groups on the protein surfaces. Such is unlikely, however, since we have identified only two specific cross-links, and they appear to represent only a single interaction. There are many surface-accessible carboxylates and amines on both MMOR-Fd and MMOH. Protein pairs that do not form tight complexes (MMOH and MMOR-FAD; MMOB and MMOR) do not cross-link under the same conditions.

**Structural Implications of the Identified Cross-links**—Because MMOR must deliver electrons to the diiron site of MMOH, it is likely that in the MMOH-MMOR complex, the [2Fe-2S] cluster of MMOH lies within about 14 Å of the diiron site to facilitate efficient electron transfer (36). The central, or canyon, region of MMOH (Fig. 5B) is the only protein surface within such a distance of the diiron center, and that region has been proposed as a binding locus for both MMOR and MMOB (16, 24). From an examination of crystal packing interactions, a model was proposed whereby MMOR could bind in the vicinity of MMOH, namely, at Lys-385. In MMOH, Lys-385 is more than 75 Å from Ala-15 (27). Even if the 14 residues between the N terminus and Ala-15 were to adopt a fully extended conformation, Ala-2 could be at most 54 Å from Ala-15. The present cross-linking results thus exclude MMOR binding in the vicinity of MMOH, Lys-385. Instead, the canyon appears to be the most likely site for MMOR binding to MMOH, a conclusion similarly reached for MMOB binding to MMOH (16). From an analysis of steady-state kinetic behavior, however, MMOB and MMOR do not appear to compete for the same binding site on MMOH (17).

Disorder at the N terminus of the α subunit in the MMOH crystal structure may suggest that this peptide fragment is unstructured. If so, then cross-linking to a specific location on MMOR-Fd might reflect only those carboxylate residues that are accessible to it upon complex formation. We must consider

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**TABLE II**

Tandem MS data for peptide with cross-link between MMOH and MMOR-Fd Glu-56 and MMOH N terminus

| Mass (obs.) | Mass (calc.) | Difference | Error | Da ppm | Structure | Ion type |
|------------|--------------|------------|-------|--------|-----------|----------|
| 1941.944   | 1941.935     | 0.009      | 5     |   | ALCSE(ALSTATK)GDYDLK | Precursor |
| 1870.931   | 1870.898     | 0.034      | 18    | LCESE(ALSTATK)GDYDLK | y_{10} |
| 1757.814   | 1757.814     | 0.000      | 2     |   | CSE(ALSTATK)GDYDLK | y_{9} |
| 1739.889   | 1739.804     | 0.085      | 7     |   | ALCSE(ALSTATK)GDYDLK | b_{9} |
| 1682.754   | 1682.754     | 0.011      | 4     |   | ALCSE(ALSTATK)GDYDLK | y_{8} |
| 1597.798   | 1597.798     | 0.009      | 6     |   | SE(ALSTATK)GDYDLK | y_{8} |
| 1510.752   | 1510.752     | 0.006      | 4     |   | BSE(ALSTATK)GDYDLK | y_{8} |
| 1498.825   | 1498.825     | 0.002      | 1     |   | CSE(ALSTATK)GDYDLK | b_{9} |
| 1322.617   | 1322.589     | 0.028      | 21    |   | ALCSEA(GDYDLK) | b_{9} |
| 1232.603   | 1232.615     | -0.012     | -10   |   | ALCSE(ALSTATK) | b_{9} |
| 1214.611   | 1214.615     | -0.005     | -4    |   | ALCSE(ALSTATK) | y_{9} |
| 1048.490   | 1048.490     | 0.020      | 19    |   | CSE(ALSTATK) | b_{9} |
| 1030.471   | 1030.480     | -0.001     | -9    |   | CSE(ALSTATK) | y_{9} |
| 709.332    | 709.328      | 0.004      | 6     |   | GDYDLK | y_{6} |
| 690.388    | 690.399      | 0.011      | -16   |   | ALSTATK | y_{7} |
| 652.298    | 652.307      | -0.009     | -14   |   | DYDLK | y_{6} |
| 619.354    | 619.354      | 0.000      | 0     |   | LSTATK | y_{6} |
| 537.291    | 537.280      | 0.011      | 20    |   | YDLK | y_{4} |
| 506.265    | 506.270      | -0.005     | -10   |   | STATK | y_{4} |
| 450.134    | 450.139      | -0.005     | -11   |   | GDYD | b_{9} |
| 419.237    | 419.238      | -0.001     | -3    |   | TATK | y_{3} |
| 383.134    | 383.117      | 0.017      | 13    |   | DPD | b_{9} |
| 374.217    | 374.217      | 0.000      | 1    |   | DLK | y_{3} |
| 344.154    | 344.160      | -0.007     | -19   |   | ALC | y_{3} |
| 315.198    | 315.190      | 0.008      | 24    |   | ATR | y_{3} |
| 278.092    | 278.090      | 0.002      | 6     |   | YD | b_{9} |
| 259.190    | 259.190      | 0.000      | 0     |   | LK | y_{2} |
| 247.153    | 247.153      | 0.000      | 1     |   | TK | y_{2} |
| 241.180    | 241.179      | 0.001      | 4     |   | LK | y_{2} |
| 184.149    | 184.129      | -0.009     | -50   |   | AL | b_{2} |
| 156.128    | 156.134      | -0.007     | -42   |   | AL | b_{2} |
| 146.104    | 146.106      | -0.002     | -14   |   | K | y_{1} |
| 132.033    | 132.030      | 0.003      | 19    |   | C | L | Immonium |
| 85.090     | 85.080       | 0.010      | 113   |   | | L | Immonium |
the possibility that the crystal structure might not reflect the structure of MMOH in complex with other proteins. It has been proposed on the basis of small angle x-ray scattering experiments that MMOH undergoes a large structural change upon formation of a ternary MMOH-MMOR-MMOR complex (14), although this result was only obtained in the presence of a
large, physiologically unrealistic excess of MMOB and MMOR. Our results suggest that the N terminus of the MMOH α subunit may become more ordered upon binding of MMOR.

*M. capsulatus* (Bath) MMOR forms cross-links to MMOH, whereas MMOR from *M. trichosporium* OB3b apparently cross-links to MMOH (11). This alternative cross-linking behavior may reflect differences in the location of reactive residues on the surfaces of the proteins from the two species. The structures of the two MMOH proteins are quite similar (6, 24), and sequence identities for MMOH, MMOH and MMOR proteins are 81, 59, and 42%, respectively (20). Taken together, these facts suggest that MMOR binds to a location on MMOH where the α and β subunits are in close proximity to one another.

NMR binding studies of MMOR-Fd and MMOH revealed specific residues on MMOR-Fd that comprise the binding surface. These residues are on the same face of the protein as the [2Fe-2S] cluster, consistent with a model in which the two proteins bind in a manner so as to bring the redox active [2Fe-2S] and carboxylate-bridged diiron centers close to one another. The locations of the MMOR-Fd β-sheet, including Glu-56 and Glu-91, experience the smallest changes in backbone 15N line widths upon binding to MMOH (7). It is not clear why Glu-56 and Glu-91, residues that form cross-links and, thus, are presumed to be involved in the binding interaction as supported by the diminution of sMMO activity in the MMOR EQ2 mutant, should appear from the NMR experiment not to be part of the binding face of MMOR-Fd.

Conclusion—Two EDC-promoted cross-links between Glu-56 and Glu-91 of MMOR-Fd and the N terminus of MMOH were identified through the use of LC-QqTOF mass spectrometry. Accurate masses combined with tandem mass spectra confirmed the structures of the cross-links. The locations of Glu-56 and Glu-91 on MMOR-Fd, close to each other on strands β_i and β_ii, suggest that the two cross-links represent a common site of interaction between the N terminus of MMOH, and the negatively charged region formed by these two carboxylates.

Mutation of full-length MMOH Glu-56 and Glu-91 both to Gln reduces sMMO activity without seriously curtailing the NADH oxidase activity of isolated MMOR. This result suggests that the interaction identified by the cross-linking study is relevant to the formation of the MMO-MMOR complex. The double mutant still forms cross-links to MMOH, indicating that other sites of cross-link formation exist and remain to be identified.

REFERENCES

1. Merkx, M., Kopp, D. A., Sazinsky, M. H., Blazyk, J. L., Muller, J., and Lippard, S. J. (2001) *Angew. Chem. Int. Ed.* 40, 2782–2807

2. Rosenzweig, A. C., Nordlund, P., Takahara, P. M., Frederick, C. A., and Lippard, S. J. (1995) *Chem. Biol.* 2, 409–418

3. Whittington, D. A., and Lippard, S. J. (2001) *J. Am. Chem. Soc.* 123, 827–838

4. Whittington, D. A., Sazinsky, M. H., and Lippard, S. J. (2001) *J. Am. Chem. Soc.* 123, 1794–1795

5. Whittington, D. A., Rosenzweig, A. C., Frederick, C. A., and Lippard, S. J. (2001) *Biochemistry* 40, 3476–3482

6. Elango, N., Radhakrishnan, R., Froland, W. A., Wallar, B. J., Earhart, C. A., Blazyk, J. L., Lippard, S. J., and Brudvig, G. W. (2002) *Biochemistry* 41, 42–51

7. Walters, K. J., Gassner, G. T., Lippard, S. J., and Wagner, G. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 7877–7882

8. Walters, K. J., Gassner, G. T., Lippard, S. J., and Wagner, G. (1999) *Biochemistry* 38, 5799–5812

9. Chang, S. L., Wallar, B. J., Lipscomb, J. D., and Mayo, K. H. (1999) *Biochemistry* 38, 6752–6760

10. Kopp, D. A., Blazyk, J. L., and Lippard, S. J. (2001) *Biochemistry* 40, 14932–14941

11. Elango, N., Radhakrishnan, R., Froland, W. A., Wallar, B. J., Earhart, C. A., Blazyk, J. L., and Lippard, S. J. (2001) *Biochemistry* 40, 15780–15794

12. Coufal, D. E., Blazyk, J. L., Whittington, D. A., Wu, W. W., Rosenzweig, A. C., and Lippard, S. J. (2000) *Biochemistry* 39, 9539–9551

13. Gallagher, S. C., Callaghan, A. J., Zhao, J. K., Dalton, H., and Trewella, J. (1999) *Biochemistry* 38, 6752–6760

14. Balandra, S., Leitner, C., Smith, T. J., and Dalton, H. (2002) *Biochemistry* 41, 2571–2579

15. MacArthur, R., Sazinsky, M. H., Kuhne, H., Whittington, D. A., Lippard, S. J., and Brudvig, G. W. (2002) *J. Am. Chem. Soc.* 124, 13392–13393

16. Gassner, G. T., and Lippard, S. J. (1999) *Biochemistry* 38, 12768–12785

17. Kang, S., Kopp, D. A., Gassner, G. T., Blazyk, J. L., and Lippard, S. J. (2001) *Biochemistry* 40, 14932–14941

18. Blazyk, J. L., and Lippard, S. J. (2002) *Biochemistry* 41, 15780–15794

19. Coufal, D. E., Blazyk, J. L., Whittington, D. A., Wu, W. W., Rosenzweig, A. C., and Lippard, S. J. (2000) *Eur. J. Biochem.* 267, 2174–2185

20. Wilkins, M. R., Lindskog, I., Gasteiger, E., Barouch, A., Sanchez, J. C., Hochstrasser, D. F., and Appel, R. D. (1997) *Electrophoresis* 18, 403–408

21. Kopp, D. A., Gassner, G. T., Blazyk, J. L., and Lippard, S. J. (2001) *Biochemistry* 40, 14932–14941

22. Peri, S., Steen, H., and Pandey, A. (2001) *Trends Biochem. Sci.* 26, 687–689

23. Perkins, D. N., Pappin, D. J., Creasy, D. M., and Cottrell, J. S. (1999) *Electrophoresis* 20, 3551–3567

24. Rosenzweig, A. C., Frederick, C. A., and Lippard, S. J., Nordlund, P. (1993) *Nature* 366, 537–543

25. Buzy, A., Miller, A. L., Legros, V., Wilkins, P. C., Dalton, H., and Jennings, K. R. (1998) *Eur. J. Biochem.* 254, 602–609

26. Correll, C. C., Batic, C. J., Balliu, D. P., and Ludwig, M. L. (1992) *Science* 258, 1604–1610

27. Rosenzweig, A. C., Brandstetter, H., Whittington, D. A., Nordlund, P., Lippard, S. J., and Frederick, C. A. (1997) *Proteins* 29, 141–152

28. Mann, M., Hendrickson, R. C., and Pandey, A. (2001) *Annu. Rev. Biochem.* 70, 437–473

29. Cai, K., Itoh, Y., and Khorana, F. C. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4877–4882

30. Itoh, Y., Cai, K., and Khorana, H. G. (2001) *Proc. Natl. Acad. Sci. U. S. A.* 98, 4883–4887

31. Alley, S. C., Trakselis, M. A., Mayer, M. U., Ishmael, F. T., Jones, A. D., and Benkovic, S. J. (2001) *J. Biol. Chem.* 276, 39340–39349

32. Ishmael, F. T., Alley, S. C., and Benkovic, S. J. (2001) *J. Biol. Chem.* 276, 25236–25241

33. Ishmael, F. T., Alley, S. C., and Benkovic, S. J. (2002) *J. Biol. Chem.* 277, 20555–20562

34. Muller, E. C., Lapko, A., Otto, A., Muller, J. J., Ruckpaul, K., and Heinemann, U. (2001) *Eur. J. Biochem.* 268, 1837–1843

35. Furukawa, Y., Matsuoka, P., Ishimori, K., and Morishima, I. (2002) *J. Am. Chem. Soc.* 124, 4008–4019

36. Page, C. C., Moser, C. C., Chen, X. X., and Dutton, P. L. (1999) *Nature* 402, 47–52
Structural Features of Covalently Cross-linked Hydroxylase and Reductase Proteins of Soluble Methane Monooxygenase as Revealed by Mass Spectrometric Analysis
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