Sulfite oxidase deficiency is a lethal genetic disease that results from defects either in the genes encoding proteins involved in molybdenum cofactor biosynthesis or in the sulfite oxidase gene itself. Several point mutations in the sulfite oxidase gene have been identified from patients suffering from this disease worldwide. Although detailed biochemical analyses have been carried out on these mutations, no structural data could be obtained because of problems in crystallizing recombinant human and rat sulfite oxidases and the failure to clone the chicken sulfite oxidase gene. We synthesized the gene for chicken sulfite oxidase de novo, working backward from the amino acid sequence of the native chicken liver enzyme by PCR amplification of a series of 72 overlapping primers. The recombinant protein displayed the characteristic absorption spectrum of sulfite oxidase and exhibited steady state and rapid kinetic parameters comparable with those of the tissue-derived enzyme. We solved the crystal structures of the wild type and the sulfite oxidase deficiency–causing R138Q (R160Q in humans) variant of recombinant chicken sulfite oxidase in the resting and sulfite-bound forms. Significant alterations in the substrate-binding pocket were detected in the structure of the mutant, and a comparison between the wild type and mutant protein revealed that the active site residue Arg-450 adopts different conformations in the presence and absence of bound sulfate. The size of the binding pocket is thereby considerably reduced, and its position relative to the cofactor is shifted, causing an increase in the distance of the sulfur atom of the bound sulfate to the molybdenum.

Sulfite oxidase (SO), an enzyme containing the molybdenum cofactor (Moco), catalyzes the oxidation of sulfite to sulfate, the final step in the degradation of sulfur-containing amino acids. It resides in the intermembrane space of mitochondria, where it exists as a homodimer. The crystal structure of chicken sulfite oxidase (CSO), purified from chicken liver, showed that each subunit contains three domains. A small heme-containing N-terminal cytochrome b6 domain (residues 3–84) is connected to the rest of the protein via a flexible 10-residue-long loop region. The central domain (residues 92–323) contains the active site of the enzyme. Finally, the C-terminal dimerization domain (residues 324–466) displays the same topology as the C2 subtype of the immunoglobulin superfamily (1).

Upon oxidation of sulfite, Mo(VI) is reduced to Mo(IV) by two electrons. The electrons are subsequently transferred to the heme Fe(III) in the cytochrome b6 domain in a two-step reaction, which is followed by transfer of the electrons from Fe(II) to cytochrome c (2). The distance between the two metals, molybdenum and iron, in the crystal structure of chicken liver sulfite oxidase (CSLO) is 32 Å, which is much longer than expected for the electron transfer rate observed (1). Two mechanisms were suggested to explain these results; a very efficient electron transfer through main chain atoms via Arg-138, a residue in the active site of the protein, or a conformational change of the b6 domain toward the molybdenum, thereby bringing the two metals into closer proximity to each other (1). Recent studies showed a dependence of the intramolecular electron transfer (IET) rate on the viscosity of the solution, supporting the idea of a possible conformational change as proposed in the second mechanism (3).

The reaction catalyzed by SO is vital for humans, because the deficiency of this enzyme leads to severe neurological abnormalities and early death (4). The symptoms of SO deficiency include dislocation of ocular lenses, mental retardation, and attenuated growth of the brain. The affected patients usually die at the age of 1 or 2 years. This deficiency may result from either a defect in Moco biosynthesis, which also affects all other molybdopterin-containing enzymes, or a defect in the sulfite oxidase gene itself. A mutation of Arg-160 to Gln in human sulfite oxidase was identified from patients suffering from this deficiency (5, 6) and was reported to cause an increase in the $K_m$ and a decrease in the $k_{cat}/K_m$ values, resulting in a 1000-fold decrease in its second-order rate constant ($k_{cat}/K_m$) (6). This residue is conserved among all sulfite oxidases sequenced to date. In the crystal structure of CLSO, the corresponding residue, Arg-138, forms the positively charged active site together with Arg-190, Arg-450, and Tyr-322 (1). Arg-138 is in close proximity to the molybdenum and forms a hydrogen bond with the water/hydroxyl ligand of the molybdenum. In addition, recent studies demonstrated that an R160Q mutation in human SO decreased the rate of intramolecular electron transfer significantly, making it the rate-limiting step in the catalytic cycle of sulfite oxidase (7). All of these studies indicate the importance of this residue for the activity of sulfite oxidase.

Although detailed structural information was obtained from the CLSO structure, no structural analysis on SO mutants that cause isolated sulfite oxidase deficiency could be obtained. Attempts to clone the chicken sulfite oxidase (CSO) gene were unsuccessful, and sequencing...
of the chicken genome has not yielded a clone for the gene yet (8). Sulfite oxidases from human (9) and rat (10) were successfully cloned and expressed, but neither of them yielded diffraction quality crystals. In the current study, we describe how crystallographic analysis on sulfite oxidase mutants that cause isolated sulfite oxidase deficiency became possible after the successful synthesis of the CSO gene, and expression of the protein in Escherichia coli. The design was based on the amino acid sequence determined by Edman degradation (11, 12) and electron density maps (1) obtained from the tissue-derived chicken sulfite oxidase. As a first attempt to gain insight into the changes caused in the sulfite oxidase-deficient mutants, we solved the structures of recombinant chicken sulfite oxidase (rCSO) and the R138Q variant of rCSO both in the resting and sulfate-bound forms.

MATERIALS AND METHODS

Synthesis of the rCSO Gene—Primer sequences for gene synthesis were based on the amino acid sequence of CLSO purified from chicken liver and utilized the codon preference of E. coli. Problematic repeats, secondary structure, and undesired restriction sites were eliminated by substituting a less preferred codon. To create the gene de novo, a total of 72 primers were obtained from Invitrogen and amplified by PCR as described previously (13). Of these primers, 36 constituted the coding strand (F1, F2, . . . ) and the other 36 made up the anticoding strand (R1, R2, . . . ). Each primer was 40 nucleotides in length with the reverse strand (F1, F2, . . . ) and the other 36 made up the anticoding strand (R1, R2, . . . ). Each primer was 40 nucleotides in length with the reverse primer offset from the forward by 20 bases (Fig. 1). The initial PCR mixture contained 1 pmol of all 72 primers, 0.2 mM dNTPs, 1.6 mM Mg2+, and 1 μl of Elongase (Invitrogen) in a volume of 50 μl. The PCR consisted of 71 cycles of 30 s at 94 °C, 30 s at 52°C, and 45 s at 68 °C. The resulting product was subjected to a second round of PCR using only those primers corresponding to the 5’ ends of the coding and anticoding strands. For this reaction, the PCR mixture contained 0.1 μl of the first PCR product, 0.2 mM dNTPs, 10 pmol of each 5’ primer, 1.4 mM Mg2+, and 1 μl of Elongase in a volume of 50 μl. PCR conditions were 20 cycles of 30 s at 94 °C, 30 s at 60 °C, and 2 min at 68 °C. The product of the second round of PCR migrated as a single band with the expected molecular weight of 1460 bp on a 1% agarose gel. This band representing the protein in

Steady State and Stopped-flow Kinetics of rCSO—Steady state kinetic assays were performed aerobically at 25 °C using a 1.0-cm path length cuvette in a Shimadzu UV-1601PC spectrophotometer. Assays were carried out in 50 mM BisTris, BisTris propane, or Tris buffers using acetic acid for pH adjustment to minimize anion inhibition (16, 17). The pH of glycine buffers was adjusted using NaOH. Sulfite oxidase concentration was determined from the A231 using a molar extinction coefficient of 113,000 per heme. The steady state pH profile was obtained using 15 μM cytochrome c, either 4.8 (wild type) or 96 nM (R138Q) rCSO, and varying concentrations of sulfite in a final sample volume of 1 ml. The reduction of cytochrome c was monitored at 550 nm using a molar extinction coefficient of 19,000. All values reported were based on the average of at least three assays.

The reductive half-reaction of rCSO was monitored using an SX.18MV stopped-flow reaction analyzer (Applied Photophysics Ltd., Surrey, UK). The dead time of the instrument was <1.7 ms with a path length of 10 mm. All assays were performed aerobically at 25 °C using 50 mM BisTris, Tris or glycine buffer, 0.5 μM enzyme, and varying concentrations of sulfite as described previously (18). Reduction of the b5-type heme Soret band was monitored at 415, 426, or 430 nm. Similar rate constants were obtained when the Ω peak was monitored at 560 nm. The kobs of each reaction was obtained by fitting the kinetic trace to a single exponential curve using a nonlinear least squares Levenberg-Marquardt algorithm. The steady state reaction parameters kcat and Km were obtained by a direct fit of the concentration dependence to the Michaelis-Menten equation. The maximal rate parameter kcat and the Km sulfate for the reductive half-reactions were obtained by fitting the kobs at varying sulfite concentrations to a hyperbolic curve as described elsewhere (17).

Mass Spectrometry of CSO—MALDI-TOF MS-MS analyses were performed at the Duke University mass spectrometry center. Samples of both tissue-derived and recombinant CSO protein (1 mg/ml) were subjected to digestion by trypsin or chymotrypsin (0.1 mg/ml) for a minimum of 1 h to a maximum of 14 h. Formic acid (to a final concentration of 0.3%) was added to quench the digestions. Zip tips were used to remove salts from the digestions, and after binding to a C-18 matrix, the protein/matrix slurries were spotted onto grids for MALDI-TOF analysis using an Applied Biosystems ABI 4700 mass spectrometer. Peptides were sequenced by ESI-MS/MS using software from BioAnalyst as described elsewhere (19).

Cryocrystallization and Data Collection—After purification, the buffer was exchanged to 10 mM Tris-Cl, pH 7.8, using a PD-10 column, and the protein was concentrated to 8 mg/ml using a Centricon YM-30 concentrator (Millipore) and stored at 4 °C prior to crystallization. Crystals of resting rCSO and rCSO-R138Q were obtained at 22°C by vapor diffusion against 1 ml of reservoir solution containing 8–12% PEG 4000, 50–60 mM MgCl2, and 100 mM MES, pH 6.5. The crystals diffracted up to 2.2 Å resolution and belong to space group I41, with a = b = 86.0 Å and c = 153.8 Å and contain one molecule in the asymmetric unit. To obtain SO42−-bound wild type crystals, 110 mM MgSO4 was used instead of MgCl2. SO42−-bound R138Q mutant crystals were obtained using similar conditions with 12% PEG 4000, 60 mM MgSO4, and 100 mM Hepes, pH 7.0. Crystals grown in MgSO4 belong to the monoclinic space group P21, with a dimer in the asymmetric unit (a = 54.0 Å, b = 123.2 Å, c = 55.5 Å, and β = 94.7°). Crystals were transferred into mother liquor containing 30% glycerol as cryoprotectant by a stepwise increase in glycerol concentration and then flash-frozen in liquid nitrogen.
FIGURE 1. Nucleotide and amino acid sequences of rCSO. The sequences of the 72 primers used to synthesize the rCSO gene are shown in alternating boldface and normal font with the name listed at the 5’ end of each forward (F) and reverse (R) primer. The protein sequence encoded by the gene is numbered at the end of each line, starting with the alanine residue after the initial methionine. The amino acids at positions 138 and 161 are outlined.
RESULTS

De Novo Synthesis of the Gene for rCSO—Because of repeated failures in a number of laboratories to clone the native CSO gene, a synthetic gene was constructed using assembly PCR as outlined by Stemmer et al. (13). In this procedure, a set of overlapping DNA primers encompassing the entire coding and noncoding strands of the desired gene are joined into contiguous strands using PCR. To use this procedure, the complete amino acid sequence of the protein must be known. In the case of CSO, information from three sources was used to determine the primary amino acid sequence that the synthesized gene would encode. By using tryptic digestion followed by Edman degradation of CSO purified from chicken liver, Guiard and Lederer (11) and Neame and Barber (12) both reported amino acid sequences for CSO (NCBI accession number P07850). More recently, we crystallized CLSO and determined the amino acid sequence from the electron density map of the enzyme (NCBI accession numbers 1SOXA and 1SOXB) (1). Although the sequences from the different groups are largely in agreement, there were several differences. In the heme domain (residues 1–84), chemical sequencing had identified an arginine at position 6, whereas in the crystal structure, the side chain at this position appeared to be that of a glutamine or glutamic acid in one monomer and was not clearly resolved in the second monomer. To clarify the identity of the residue at this position, the same sample of liver-derived CSO used for crystallization was subjected to N-terminal sequencing. This analysis showed conclusively that residue 6 is an arginine (data not shown). In the crystal structure of the hinge region (residues 85–95), residue 89 in one monomer and residues 86–91 in the second monomer were only weakly defined and were not included in the crystal structure refinement (1).

Once the desired primary amino acid sequence for CSO was determined, a nucleotide sequence capable of encoding the amino acid sequence was formulated. Because the protein was to be expressed in an E. coli strain, the nucleotide sequence chosen used the preferred E. coli codons whenever possible. As seen in Fig. 1, 72 single-stranded DNA primers corresponding to both strands of the entire formulated nucleotide sequence were then commercially prepared. Each of these primers was 40 bases long and was designed so that all 36 forward primers corresponding to the coding strand overlapped the reverse primers constituting the noncoding strand by 20 bases. PCR amplification of a reaction mixture containing all 72 primers resulted in a single, double-stranded DNA fragment with the expected size of 1460 bp. This fragment was then sequenced to verify that it represented a full-length synthetic gene for rCSO. After correction of a number of errors introduced during the PCR process, the gene was subcloned into the plasmid pUC19 (14).

TABLE ONE

| pH     | Buffer         | $k_{cat}$ $s^{-1}$ | $K_{m,sulfite}$ $\mu M$ | $k_{cat}/K_{m,sulfite}$ $s^{-1} \mu M^{-1}$ |
|--------|----------------|-------------------|--------------------------|-------------------------------------------|
| 6.00   | Bis-Tris       | 30.8 ± 2.0        | 1.92 ± 0.26              | 1.68 × 10^7                             |
| 6.50   | Bis-Tris       | 40.9 ± 0.5        | 1.44 ± 0.06              | 2.96 × 10^7                             |
| 6.75   | Bis-Tris propane| 22.6 ± 0.6        | 1.11 ± 0.08              | 2.11 × 10^7                             |
| 7.00   | Bis-Tris propane| 35.9 ± 0.1        | 1.28 ± 0.08              | 2.93 × 10^7                             |
| 7.50   | Bis-Tris propane| 46.2 ± 1.9        | 1.92 ± 0.16              | 2.50 × 10^7                             |
| 7.50   | Tris           | 58.5 ± 0.4        | 1.92 ± 0.11              | 3.18 × 10^7                             |
| 8.00   | Tris           | 66.0 ± 2.2        | 3.94 ± 0.16              | 1.74 × 10^7                             |
| 8.25   | Tris           | 74.5 ± 1.8        | 5.09 ± 0.34              | 1.52 × 10^7                             |
| 8.50   | Tris           | 73.3 ± 6.7        | 8.43 ± 1.58              | 9.16 × 10^6                             |
| 8.75   | Tris           | 75.8 ± 3.5        | 11.9 ± 0.8               | 6.62 × 10^6                             |
| 8.75   | Glycine        | 72.4 ± 2.3        | 15.1 ± 1.4               | 5.01 × 10^6                             |
| 9.00   | Glycine        | 70.5 ± 1.5        | 22.5 ± 2.2               | 3.28 × 10^6                             |
| 9.50   | Glycine        | 62.3 ± 2.2        | 53.8 ± 9.4               | 1.23 × 10^6                             |
| 10.0   | Glycine        | 23.5 ± 0.6        | 39.2 ± 7.7               | 6.37 × 10^5                             |
Analysis of rCSO—Both untagged and His\textsubscript{6}-tagged rCSO proteins were expressed and purified using conditions optimized for recombinant SO from other species (14). Although little His\textsubscript{6}-tagged rCSO was produced, expression of untagged rCSO from the synthetic gene was comparable with that observed previously using the same expression system with recombinant human SO (14). The oxidized and sulfite-reduced absorption spectra of purified rCSO are shown in Fig. 2. They look identical to that observed for CLSO purified from liver (24), with the characteristic 5 cytochrome bands evident in both. The molybdenum content in all preparations of purified rCSO averaged 0.75 mol/mol of subunit as determined by atomic absorption spectrometry. The values reported for $k_{\text{cat}}$ are normalized to a molybdenum content of 100% and agree well with those published in earlier studies using CLSO (3, 17). The slightly lower activity observed in samples assayed in BisTris propane buffer can be attributed to the higher concentration of acetate ions in this buffer system. The results of rapid kinetic assays using stopped-flow analysis of rCSO at several pH values are summarized in TABLE TWO. In assays of the reductive half-reaction, $k_{\text{cat}}$ changed very little over the pH range, whereas $K_{d,\text{red}}$ increased with higher pH values from 3.17 μM at pH 7.0 to 268 μM at pH 9.8. Again, these results are consistent with previously published rapid kinetic studies using CLSO (17).

Structure Determination—We solved the crystal structures of both wild type rCSO and the R138Q mutant in the resting and sulfate-bound forms by molecular replacement using the CLSO coordinates (monomer-residues 95–466, Protein Data Bank entry 1SOX) as the search model. Although full-length rCSO was used for crystallization, no difference electron density ($F_{\text{o}} - F_{\text{c}}$, map) corresponding to the N-terminal $b_\text{5}$ domain (residues 1–94) was observed in any of the structures we obtained. This might be due to proteolytic cleavage of the N-terminal domain and selective crystallization of the catalytic core and the C-terminal dimerization domain. Similar results were observed in the crystallization trials of full-length human SO, where only crystals of the N-terminal $b_\text{5}$ domain were obtained (25).

Crystals of the resting rCSO and rCSO-R138Q mutant belong to space group $I_4_1$ with one monomer in the asymmetric unit, whereas the sulfate-bound rCSO and the rCSO-R138Q variant were crystallized in space group $P_2_1$ with a dimer in the asymmetric unit. The sulfate-bound rCSO contains two sulfate molecules per monomer, whereas the R138Q variant only contains one. All structures have good stereochemistry with all residues in the most favored and additionally allowed regions of the Ramachandran diagram as determined by PROCHECK (26) (TABLE THREE).

Structure of rCSO—The structure of rCSO contains two domains, the catalytic core domain and the C-terminal dimerization domain (Fig. 3). As described earlier, the catalytic core domain has a unique fold composed of 3 β-sheets with a total of 13 β-strands and 9 α-helices (1). The Moco is located at the center of this domain and is held tightly in place through 16 hydrogen bonds with the protein backbone and through side chains and numerous van der Waals contacts.

Comparison of the rCSO and CLSO Structures—A superposition of the structures of rCSO and CLSO shows that they are almost identical as indicated by a root mean square (r.m.s.) deviation of 0.33 Å for the C-α atoms of all residues in the structure of rCSO (95–466). However, a

TABLE TWO

| pH | $k_{\text{red}}$ (μM) | $K_{d,\text{red}}$ (μM) | $k_{\text{red}}/K_{d,\text{red}}$ |
|----|------------------|------------------|------------------|
| 7.0 | 1160.8           | 3.17             | $5.07 \times 10^7$ |
| 8.0 | 180.8            | 9.50             | $1.90 \times 10^8$ |
| 8.3 | 183.1            | 18.6             | $9.84 \times 10^5$ |
| 9.0 | 193.2            | 65.7             | $2.94 \times 10^9$ |
| 9.8 | 208.9            | 268              | $7.79 \times 10^9$ |

TABLE THREE

| Crystallographic statistics | Wild type rCSO resting state | Sulfate-bound rCSO | R138Q mutant resting state | Sulfate-bound R138Q mutant | Sulfate-bound Arg-161 rCSO |
|-----------------------------|-------------------------------|-------------------|---------------------------|---------------------------|---------------------------|
| $R_{\text{sym}}$ | 0.08 (0.60) | 0.10 (0.55) | 0.09 (0.60) | 0.16 (0.52) | 0.07 (0.38) |
| Residues | 95–466 | (96–466), (108–466) | 95–466 | (108–466), (108–466) | 95–158, 162–466, (108–466) |
| No. protein/cofactor atoms | 2900 | 5689 | 2895 | 5597 | 5697 |
| No. waters | 235 | 655 | 214 | 529 | 822 |
| $R_{\text{cryst}}$ ($R_{\text{free}}$) | 0.15 (0.18) | 0.16 (0.22) | 0.16 (0.20) | 0.16 (0.24) | 0.16 (0.19) |
| r.m.s. deviation bond lengths (Å) | 0.014 | 0.016 | 0.010 | 0.015 | 0.012 |
| Mean B-factor | 45.2 | 22.0 | 42.9 | 19.6 | 20.8 |
| Ramachandran statistics | 93.0/7.0 | 90.8/9.0 | 90.3/9.7 | 90.7/9.1 | 90.2/9.8 |
small loop region around residue 161 adopted a different conformation. This residue was shown to be an arginine in the protein sequence determined by Edman degradation (12), and it was assigned as a glycine from the electron density map obtained from the crystals of CLSO (1). Initially, we placed a glycine at this position during the synthesis of the rCSO gene. To test if the difference between the structures of rCSO and CLSO is because of this change in the amino acid sequence at position 161, the CSO amino acid sequence was reexamined through MALDI-based analysis of tryptic and chymotryptic peptides of native CLSO. All but one of the peptides generated by these digestions that were large enough to be observed displayed masses completely in agreement with the sequence used for the synthetic gene. The one exception was a trypic peptide representing residues 153–171, the sequence of which was interpreted to be LRVDGPGRTLSLSLAELR. This result indicated that the crystallographic assignment of residue 161 as a glycine was erroneous and that the residue is in fact an arginine.

Based on these findings, the codon for residue 161 was modified to encode an Arg, and the modified gene was expressed in E. coli. Purification of Arg-161 rCSO yielded a protein with spectral properties identical to those of Gly-161 rCSO and native forms of CSO (data not shown). The molybdenum content was determined to be 0.78 mol/mol of CSO. Kinetic values were also similar to both Gly-161 rCSO and native forms of CSO, with a $k_{\text{cat}}$ of 63.9 ± 5.2 s$^{-1}$ and a $K_m$ for sulfite of 14.2 ± 6.7 μM using 50 mM Tris acetate buffer, pH 8.5. Furthermore, the resulting protein, Arg-161 rCSO, was crystallized. In the structure of Arg-161 rCSO, the region around residue 161 in one of the monomers adopted the same conformation as the nonrecombinant enzyme, confirming that this residue is actually an arginine, whereas this region is disordered in the second monomer. However, this change in amino acid sequence of rCSO affected neither the structure around the active site nor the activity of the enzyme. Hence we used the data obtained from rCSO with a glycine at residue 161 for further discussion because we could not obtain high-quality crystals for the resting state of Arg-161 rCSO.

Comparison of the Structures of Resting and Sulfate-bound rCSO—

We obtained crystals under two different conditions, one with bound sulfate and another without bound substrate or product. In the crystals that were obtained in the presence of sulfate, we observed two sulfate molecules per monomer at the same locations as observed previously in the CLSO structure; one was in the active site and another was around the opening of the active site. The sulfate molecule in the active site is coordinated by a dense hydrogen bond network between the oxygens of the sulfate and the side chains of the active site residues Arg-190, Arg-450, Tyr-322, Trp-204, and the main chain nitrogen atom of Leu-202 (Fig. 4). Crystallization media that did not contain sulfate yielded crystals of the resting enzyme with one chloride ion in the active site. Initially, we placed a water molecule at this position, but due to the presence of additional $F_o - F_c$ density and a low B-factor for the water molecule, we concluded that this is a chloride ion. The chloride ion is coordinated by active site residues Arg-190, Trp-204, and two water molecules. Superposition of the monomers from both structures reveals that there is no significant change in the overall structure of the catalytic and dimerization domains. A rigid body movement for the N-terminal $b_5$ domain was suggested upon sulfate binding (27), but no information regarding this movement could be obtained because this domain is not present in any of the new rCSO structures.

A closer look at the active sites of these two structures indicates a conformational change of the side chain of Arg-450. In the sulfate-bound structure, the side chain of Arg-450 points toward the active site coordinating the sulfate molecule with its guanidinium group, and it points toward the solvent in the structure of the resting enzyme (Fig. 4). The same change in the orientation of the side chain of this residue was observed between the sulfate-bound structure of CLSO and the sulfate-bound structure of recombinant chicken sulfite oxidase.
Structure of Recombinant Chicken Sulfite Oxidase

The resting rCSO crystallized with a monomer in the asymmetric unit, but the dimer is generated by crystallographic symmetry as expected. Superposition of the monomers of resting and sulfate-bound structures revealed a slight rotation of the molybdopterin-containing domain of the other monomer. This rotation is not as significant as the one observed in the comparison of the sulfate-free structure of plant sulfite oxidase and sulfate-bound CLSO, and does not lead to any change in the distance of the two molybdenum atoms of the dimer.

The Sulfite Oxidase Deficiency Causing rCSO-R138Q Mutant—The R160Q variant of human SO was one of the first to be identified in a patient with SO deficiency (6). Arg-138 in CSO is equivalent to Arg-160 in human SO, so the R138Q rCSO variant was generated by site-directed mutagenesis of the rCSO gene, expressed in E. coli, and purified using the same protocol as for wild type rCSO. The protein expressed well, was amenable to purification, and contained 82% of a full complement of molybdenum. When steady state assays were performed on R138Q rCSO under standard conditions of 50 mM Tris acetate, pH 8.5, it was significantly impaired in catalysis ($k_{\text{cat}} = 3.4 \text{ s}^{-1}$) as well as sulfite binding ($K_m = 0.25 \text{ mM}$). Using identical assay conditions, recombinant R160Q human SO yielded a $k_{\text{cat}}$ of 3.3 s$^{-1}$ with a $K_m$ for sulfite of 0.85 mM.

The structure of the R138Q mutant was refined to 2.5 Å resolution with an $R$-factor of 0.16 and an $R_{\text{free}}$ of 0.20. The structure of this SO deficiency-causing variant is identical to the wild type structure as shown by the low r.m.s. deviation of 0.16 Å for the C-α atoms of all residues present in both structures (Fig. 5A). The active site residues Arg-190, Tyr-322, Cys-185, and Trp-204 adopt the same conformations in both structures (Fig. 5B). The electron density corresponding to the Moco is well defined and indicates no differences in both structures. Furthermore, the water/hydroxo ligand of the molybdenum is at the same position in the mutant, forming a hydrogen bond with the side chain of Gln-138 instead of Arg-138. In the structure of the resting R138Q mutant structure, however, Arg-450 is oriented toward the active site as seen for the sulfate-bound wild type protein, yet no sulfate is present (Fig. 5B). This movement of Arg-450 with the gap created because of the shorter side chain of glutamine creates a shift in the position of the substrate binding pocket (Fig. 5, C and D). Because of this conformational change, the binding pocket is paradoxically smaller in the mutant, although a smaller residue (Gln) has replaced a larger residue (Arg).

As expected, the electrostatic surface potential around the active site is changed by the mutation of the positively charged arginine to a neutral glutamine. However, a more dramatic change in the surface potential is observed because of the conformational change of the side chain of Arg-450. Asp-321, which is buried by Arg-450 in the wild type protein, is solvent-exposed in the mutant (Fig. 6).

We also solved the structure of the SO deficiency variant in the presence of sulfate and observed that the distance of the sulfate to the molybdenum increases by 1.3 Å compared with the sulfate molecule in the active site of the wild type protein (Fig. 7). The sulfate molecule is coordinated by hydrogen bonds to Arg-450, Gln-138, Tyr-322, and the backbone of Leu-202. Unlike the wild type structure, it does not interact with Arg-190 and Trp-204, although the conformations of these residues are maintained. The electron density corresponding to the sulfate...
The catalytic cycle of sulfite oxidase can be divided into the following three stages: oxidation of sulfite, transfer of electrons from the molybdenum to the iron (intramolecular electron transfer), and transfer of electrons from the iron to cytochrome c. The initial event, the binding of sulfite and its oxidation to sulfate, is depicted in Fig. 8. Previous studies have shown that the binding of sulfite and its oxidation to sulfate is an essential step in the catalytic cycle of sulfite oxidase.

The active site of SO features the positively charged residues Arg-138, Arg-190, and Arg-450, presumably to attract the negatively charged substrate, sulfite, or the product, sulfate. Arg-450 stands out from the other active site residues, being the only residue that undergoes a conformational change upon sulfate binding. In the resting state of the enzyme, it points toward the solvent; after binding of sulfate, it moves toward the active site and coordinates the sulfate molecule. The structure of the resting state of the enzyme contains a chloride ion at the active site. This is not surprising considering the highly positively charged active site to attract sulfite molecules. It also explains the inhibitory effect of small anions such as chloride on sulfite oxidase (27, 29).

Biochemical studies on the R160Q mutant of human sulfite oxidase, which corresponds to R138Q in CLSO, showed that substrate binding and the catalytic activity of the enzyme were significantly impaired causing the severe phenotype observed in patients carrying this mutation (6). Because of the close proximity of this residue to the molybdenum and the differences between the absorption spectrum of the wild type and the mutant, it was argued that mutation of Arg-160 to Gln might lead to alterations in which the molybdenum is coordinated differently compared with the wild type protein; however, in the structure of the R138Q mutant, no change in cofactor binding was observed, and the well defined electron density maps around the cofactor suggest that the active site is fully occupied. The hydrogen bonds between the cofactor and the amino acids are maintained in the mutant, and the molybdenum is coordinated in the same way as in the wild type protein. The position of the water/hydroxo ligand relative to the cofactor remained the same in the mutant, and hydrogen bonding of the water/hydroxo ligand with Arg-138 was maintained with the side chain of glutamine, albeit at a longer distance, thus forming a weaker hydrogen bond compared with the wild type protein.

The most significant change in the R138Q mutant is the orientation of the side chain of Arg-450 toward the active site in the absence of the bound sulfate. This move is likely to be a result of the loss of a positive charge on residue 138 in the active site, which otherwise repels the positively charged Arg-450. This conformational change decreases the size of the binding pocket (Fig. 5). Another change in the active site due to this mutation is the shift of the binding pocket toward Gln-138, due to both a decrease in the size of the side chain of residue 138 and the movement of the side chain of Arg-450. In addition to these changes in the geometry of the binding pocket, the electrostatic potential of this pocket is less positively charged compared with the wild type protein as a result of the loss of a positive charge in the active site as well as the modified position of Arg-450, thereby exposing Asp-321 to the solvent and introducing a negative charge around the active site. The shift of the binding pocket described above is also underscored by the structure of the R138Q variant in the presence of the product sulfate. The sulfate molecule is positioned closer to Gln-138, in a way that is not possible in the wild type protein due to steric hindrance with the longer side chain of the arginine. This modified position of the sulfate molecule increases the distance of the sulfur atom to the molybdenum by 1.3 Å.

The first opportunity to recreate pathogenic human SO variants in an SO species that yields diffraction quality crystals for high resolution structural analysis. The crystallographic and kinetic analysis of wild type rCSO and the R138Q variant (analogous to the pathological R160Q human variant) described here represent the first structure-function analysis of this essential protein.

Structural studies of the recombinant chicken sulfite oxidase, which produce large quantities of functional CSO from a cloned gene, provide a more complete understanding of this important enzyme. The ability to produce large quantities of functional CSO from a cloned gene provides the first opportunity to recreate pathogenic human SO variants in an SO species that yields diffraction quality crystals for high resolution structural analysis. The crystallographic and kinetic analysis of wild type rCSO and the R138Q variant (analogous to the pathological R160Q human variant) described here represent the first structure-function analysis of this essential protein.
have implicated Arg-138 in attracting sulfite to the binding pocket. Oxidation is accomplished through bridging of the sulfur atom of sulfite to the molybdenum atom via the equatorial oxo ligand of the metal and concomitant reduction of Mo(VI) to Mo(IV). The distance between the molybdenum atom and the sulfur atom of the nascent sulfate is expected to be about 3 Å. The data presented here show that the side chain of Arg-450 in the resting wild type enzyme faces away from the binding pocket and is unlikely to be involved in the initial coordination of sulfite or the nascent sulfate. This conclusion is supported by the finding that mutation of Arg-450 to Gln, although greatly attenuating $k_{cat}$, has no effect at all on $K_m$ for sulfite, in contrast to the marked change observed in the R138Q mutant. 4 If the incoming sulfite molecule is bound in the same way as the sulfite in the structure of the sulfate-bound SO, it would be too far away from the Moco for efficient catalysis. A rational explanation of the conformational changes in the side chain of Arg-450 is that it initially faces the solvent but then turns toward the binding pocket after the formation of sulfate, and in fact facilitates the dissociation of the newly formed sulfite from the molybdenum atom. It is also possible that Arg-450 then undergoes another conformational change and assists in the departure of the product from the binding pocket into the solvent. The dissociation of the product would then be followed by electron transfer from the molybdenum atom to the heme.

In the structure of the resting R138Q mutant, the side chain of Arg-450 points toward the binding pocket in contrast to the resting state of native SO. It thus seems very likely that Arg-450 is involved in binding the incoming sulfite molecule in the R138Q variant as observed in the sulfate complex, and thus greatly hinders the formation of the bridged complex between the sulfite and the molybdenum atom. By the same token, mutation of Arg-450 to Gln will negate the proposed role of Arg-450 in product release, thus affecting the $k_{cat}$.

In a laser-flash photolysis experiment that analyzed the reverse IET from the heme to the molybdenum, it was found that the rate of IET is attenuated in the R138Q mutant (7). It was argued that this residue contributes to the proper orientation of the $b_6$ domain relative to the rest of the protein during electron transfer to allow a closer approach of the two metals, iron and molybdenum, and the loss of the positive charge at this position is responsible for the effect on IET. Our structural analysis suggests that it is not only the loss of the positive charge but also the conformational change of the side chain of Arg-450 that could be responsible for the inhibition of the proposed interaction of the $b_6$ domain with the active site of the enzyme. This interaction may require the conformation of Arg-450 observed in the resting state of the protein, in which the side chain of Arg-450 points toward the solvent and is accessible for these interactions. This conclusion is supported by the finding that the rate of IET in native SO is decreased in the presence of sulfate, which causes a change in the orientation of Arg-450. An additional effect is the introduction of a negative charge at the surface because Asp-321 becomes solvent-exposed when Arg-450 points toward the substrate binding pocket, which may have an added inhibitory effect on the IET.

In both studies that showed changes in the kinetic parameters and in the rate of the intramolecular electron transfer, mutation of Arg-138 to a lysine yielded an enzyme with parameters that are intermediate between those of the wild type and the R138Q mutant (6, 7). The positive charge of the lysine residue would repel Arg-450, providing a conformation closer to the wild type protein. In addition, the positioning of the binding pocket relative to the molybdenum is also likely to remain close to that of the wild type protein, because the length of a lysine is shorter than that of a arginine. The increased rate of the intramolecular electron transfer relative to the rate in the R138Q variant could also be the result of the introduced positive charge and the conformation of Arg-450. However, the proper orientation of sulfite may require the guanidinium group of Arg-138, resulting in a less active R138K mutant compared with the wild type protein.

In conclusion, the severe phenotype observed in the R160Q mutant is a result of the modifications on the binding site of sulfite. The size of the binding pocket is reduced significantly, and its position relative to the cofactor is shifted causing an increase in the distance of the sulfur atom of the bound sulfate to the molybdenum. These modifications are mainly because of the conformational change of Arg-450, a conserved residue that adopts different conformations upon sulfite binding.

Acknowledgments—We gratefully acknowledge the technical assistance of Ralph D. Wiley, and we thank Drs. Jean L. Johnson, Kimberly Johnson Nelson, and Margot M. Wuebbens for helpful discussions. We also thank Dr. Timothy Haystead and members of his laboratory for their valuable discussions and expertise in performing the mass spectrometry experiments and Dr. John H. Enemark and members of his laboratory for providing CLSO.

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J. Biol. Chem. 2005, 280:33506-33515.
doi: 10.1074/jbc.M505035200 originally published online July 27, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M505035200

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