Identification of Amino Acids of Influenza Virus HA Responsible for Resistance to a Fusion Inhibitor, Stachyflin

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Abstract: We have recently described a novel hemagglutinin (HA) conformational change inhibitor of human influenza virus, Stachyflin (Yoshimoto et al., Arch. Virol., 144, 1-14, 1999). Stachyflin-resistant variants of human influenza A/WSN/33 (H1N1) virus were isolated in vitro and the nucleotide sequences of their HA genes were determined. The relation of amino acid substitutions and Stachyflin resistance was analyzed with in vitro membrane fusion between HA-expressing cells and octadecylrhodamine (R18)-labelled chick erythrocytes (RBC). The amino acid substitutions, lysine to arginine at position 51 or lysine to glutamic acid at position 121 of the HA2 subunit of the HA protein was enough to confer a Stachyflin-resistant phenotype of HA protein. The molecular mechanism of anti-HA conformational change activity of Stachyflin is discussed.

Key words: Influenza virus, HA, Fusion inhibitor

Infection by enveloped viruses involves a virus-cell membrane fusion process with subsequent transfer of viral genomes into the host cells. In the influenza virus, the hemagglutinin (HA), a trimeric protein embedded in the viral membrane, mediates the fusion of viral and cellular membranes. The HA is a trimer of identical subunits and composed of two disulfide-linked subunits denoted HA1 and HA2, which are formed by the proteolytic cleavage of a precursor, HA0 (26). The conformation of the precursor, HA0, is stable at low pH, but cleaved HA is apparently metastable (2). After entry into the host cell, the virus particle encounters an acidic environment of the endosome. Extensive and irreversible conformational change is induced by the low-pH condition. It includes a dramatic refolding of HA2, where the N-terminal fusion peptide moves at least 100 Å toward the tip of the molecule (2, 3). This process facilitates insertion of the fusion peptide into the endosomal membrane.

Recently, several small molecules which inhibit the low-pH-induced HA conformational change have been reported (1, 6, 11, 16, 25, 27). In previous reports, we presented that Stachyflin was a human influenza A virus-specific virucide that inhibits low-pH-induced HA conformational change (8, 27). Currently, we have isolated Stachyflin-resistant human influenza A/WSN/33 (H1N1) virus clones and identified the amino acids of HA proteins responsible for the Stachyflin-resistant phenotype. The possible role of these amino acids in the low-pH-induced HA conformational change, molecular mechanism of Stachyflin action, and amino acid substitutions in acquiring the Stachyflin-resistant phenotype are discussed.

Materials and Methods

Cell cultures and compounds. Madin-Darby bovine kidney (MDBK) cells were grown in a solution consisting of Ham’s F-12 medium (Dainippon Pharmaceutical, Osaka, Japan) and Eagle’s minimum essential medium (E-MEM) (Biken, Osaka, Japan) at 1:1, supplemented with 10% fetal bovine serum (FBS) (Intergen, N.Y., U.S.A.). Madin-Darby canine kidney (MDCK) cells were grown in E-MEM supplemented with 10% FBS. Two-hundred-and-ninety-three EBNA 1 cells

Abbreviations: BSA, bovine serum albumin; E-MEM, Eagle’s minimum essential medium; FBS, fetal bovine serum; HA, hemagglutinin; IC₅₀, 50% inhibitory concentration; MDCK, Madin-Darby canine kidney; MTI, 3-(4,5-diethylthiazol-2-y1)-2,5-diphenyl-2H-tetrazolium bromide; NA, neuraminidase; R18, octadecylrhodamine; RBC, chick erythrocytes; TCID₅₀, tissue culture infective dose, vRNA, viral RNA.
(Invitrogen, San Diego, Calif., U.S.A.) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS. After virus infection, these cells were maintained in E-MEM containing 0.5% bovine serum albumin (BSA). A/WSN/33 (H1N1) virus was grown in 11-day-old hen eggs. Stachyflin-resistant A/WSN/33 virus clones were grown in MDCK cells. The isolation and purification of Stachyflin were previously described (27).

**Isolation and characterization of Stachyflin-resistant A/WSN/33 virus clones.** A/WSN/33 virus was plaque purified on MDCK cells. Two independent clones were amplified in MDCK cells and used to infect cells at a m.o.i. of 0.01 in the presence of Stachyflin at 0.01 µg/ml, which was a 5-fold higher concentration of 50% inhibitory concentration (IC₅₀) against A/WSN/33 virus. Viruses were serially passaged under the same condition described above. After the third passage, viruses were subjected to a further three passages in the presence of Stachyflin at 0.05 µg/ml. After the sixth passage, viruses were plaque purified on MDCK cells in the presence of Stachyflin at 0.01 µg/ml, and each virus clone was amplified in MDCK cells in the presence of Stachyflin at 10 µg/ml. A Stachyflin-resistant phenotype for each virus clone was confirmed by antiviral assay using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay as previously described (24). The EC₅₀ values were calculated according to Reed and Muench (18). Hemolysis assay and hemolysis inhibition assay were performed as described previously (25). Briefly, a 1 X 10⁷ 50% tissue culture infective dose (TCID₅₀) of virus was incubated with 1% (W/V) chick red blood cells (RBC) (Nikken, Kyoto, Japan) in 100 µl of PBS containing an appropriate concentration of Stachyflin for 15 min at 37 C followed by the induction of hemolysis with 0.1 M acetate buffer (pH 5.0). To determine the optimum pH of wild-type and mutant A/WSN/33 viruses for RBC hemolysis, the pH 5.0 buffer was replaced by buffers at pH 3.6, 3.8, 4.0, 4.2, 4.4, 4.6, 4.8, 5.2 and 5.4 (adjusted to the appropriate pH with HCl). After 30-min incubation at 37 C, the reaction mixtures were centrifuged at 1,200 rpm for 5 min and the amounts of hemoglobin in the supernatant were determined by measuring OD₅₆₀ referenced with OD₆₅₀. The IC₅₀ of Stachyflin in the hemolysis assay against Stachyflin-resistant A/WSN/33 virus clones was calculated as described previously (18).

**Nucleotide sequences of HA genes.** Viral RNA (vRNA) was extracted from cell-free viruses as previously described (9). One microgram of vRNA was converted to plus-strand cDNA using 10 pmol of HA-specific primer and *Avian myeloblastosis* virus reverse transcriptase according to the manufacturer's protocol (Gibco BRL, Rockville, Md., U.S.A.). The primer pair HA-SH: 5'GCGTGCACACAAATGAAGGCACACTACG-3' and HA-AT: 5'-GATGGGCGCGCTCAGATG-CATATTCTGCAC-3', corresponding to the nucleotide positions 27–51 and 1712–1731 of the HA gene, respectively, were used for amplification of the sequences containing the entire open reading frame of the HA gene. The cDNAs were amplified by polymerase chain reaction containing 5 µl of plus-strand cDNA, 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 200 mM dNTP, a 250 nM concentration of primers and 2.5 U of Taq DNA polymerase (Gibco BRL) in a total volume of 100 µl. The thermodenaturase profile used was as follows: heating at 95 C for 5 min followed by 35 cycles at 94 C for 1 min, 55 C for 1 min, and 72 C for 1 min. The PCR products were purified with Wizard PCR preps (Promega, Madison, Wisc., U.S.A.) and inserted into the TA cloning vector (Invitrogen). Three independent cDNA clones from each Stachyflin-resistant virus clone were sequenced using the dideoxynucleotide chain termination method (20).

**Construction of the plasmids and expression of HAs in 293 EBNA I cells.** The cDNAs encoding the entire wild-type or mutant A/WSN/33 virus HAs were made from cell-free wild-type or Stachyflin-resistant A/WSN/33 viruses by the RT-PCR technique using synthetic HA-specific oligonucleotide primers having NotI or SalI sites, respectively. To confirm the correct sequences, the PCR products digested with restriction enzymes NotI and SalI were cloned into the multi-cloning site of pBluescript II (Stratagene, La Jolla, Calif., U.S.A.) and sequenced. The mutant cDNAs possessing amino acid substitutions K51R, K121E, V176I or S206L were synthesized in vitro (20). The 293 EBNA I cells were transfected with 1 µg of plasmid DNA containing wild-type or mutant HA genes as described previously (7). Twenty-four hours after transfection, cells were treated with Vibrio cholerae neuraminidase (NA) (Sigma, 10 U/ml) and acetylated trypsin (Sigma, 4 µg/ml) for 1 hr at 37 C, followed by the addition of RBCs labelled with R18 (R-RBCs) and further incubated for 1 hr at 4 C. HA-mediated membrane fusion was induced with a low-pH buffer (pH 5.0) in the presence or absence of 10 µg/ml Stachyflin (27). The occurrence of membrane fusion was judged by the diffusion of R18 from the membranes of R-RBCs into those of the 293 EBNA 1 cells under confocal laser
microscopy as previously described (21). The R-RBCs were prepared by a previously described method (14).

Results

Isolation and Biological Characterization of Stachyflin-Resistant Influenza A/WSN/33 Virus Clones

Fourteen Stachyflin-resistant A/WSN/33 virus clones were isolated from 2 clones of wild-type A/WSN/33 (H1N1) virus by in vitro serial passages in MDCK cells in the presence of the appropriate concentration of Stachyflin, as described in “Materials and Methods.” All of the plaque-purified Stachyflin-resistant virus clones could be amplified in MDCK cells for biological characterization except for 2 clones. These 2 virus clones, designated R1 type with a single amino acid substitution, Lys to Arg, at position 51 (K51R) did not grow enough in MDCK cells for biological characterization (Tables 1, 2). Therefore, we have examined 12 other Stachyflin-resistant virus clones.

To confirm the Stachyflin-resistant phenotype of these A/WSN/33 virus clones, their susceptibility to Stachyflin was evaluated in vitro by MTT assay and RBC hemolysis inhibition assay (Table 1). None of Stachyflin-resistant virus clones examined was inhibited even at 20 μg/ml of Stachyflin, which was 10,000 times (MTT assay) or 1,000 times (RBC hemolysis inhibition assay) higher than the IC₅₀ values against the wild-type A/WSN/33 virus used in our laboratory. The pH at which 50% RBC hemolysis was observed for the wild-type virus was 4.9, and elevation of optimum pH was not seen for any Stachyflin-resistant mutants.

Nucleotide Sequence Analyses of Stachyflin-Resistant A/WSN/33 Virus Clones

Nucleotide sequences of the HA genes of wild-type and Stachyflin-resistant A/WSN/33 virus clones were determined using cDNA clones amplified by PCR from cell-free virus, and predicted amino acid sequences of HA protein were compared with that of a wild-type A/WSN/33 virus clone (Table 2). There existed four amino acid substitutions (V176I located in the HA1 subunit and K51R, K121E or S206L in the HA2 subunit, respectively) in the HA proteins of the Stachyflin-resistant A/WSN/33 virus clones. Of these amino acid substitutions, K51R was the only amino acid substitution common to all of the Stachyflin-resistant A/WSN/33 virus clones sequenced (Table 2). There was no difference in the nucleotide sequences of the HA gene in the two wild-type virus clones used in our study. However, compared to the published nucleotide sequences of the HA gene of prototype A/WSN/33 virus (5), there were several point mutations which resulted in amino acid substitutions in both HA1 and HA2 subunits (data not shown).
Effect of Amino Acid Substitutions on HA-Mediated Membrane Fusion In Vitro

The cloned cDNAs of wild-type or mutant HAs with each single amino acid substitution or all four amino acid substitutions were ligated under the EF1-
α promoter of the plasmid pDREF-His D, which was reported to have powerful performance in mammalian cells (Fig. 1) (14). The 293 EBNA 1 cells were transfected with plasmids containing cDNAs of wild-type or mutant HAs and treated with trypsin and NA 24 hr after transfection. Membrane fusion between HA-expressing cells and R-RBCs was induced by low-pH buffer. The diffusion of R18 from the membranes of R-RBCs to those of HA-expressing 293 EBNA 1 cells was readily detected 24 hr after transfection under confocal laser microscopy (Fig. 2). Membrane fusion of the R-RBCs and 293 EBNA 1 cells transfected with plasmids containing mutant HAs having amino acid substitutions K51R, K121E or all four amino acid substitutions (M2-51, M2-121 and R4, Fig. 1) was observed even in the presence of a high dose of Stachyflin (Fig. 2). However, single amino acid substitution V176L or S206L (M1-176 and M2-206) was still sensitive to Stachyflin. From these results, we have concluded that all of the amino acid substitutions observed in Stachyflin-resistant A/WSN/33 virus HAs were functional in the in vitro HA-mediated membrane fusion process and amino acid substitution K51R or K121E directly conferred Stachyflin-resistant phenotype on HA protein.

Discussion

In this study, we have isolated Stachyflin-resistant variants of A/WSN/33 (H1N1) virus and identified the amino acids of HA proteins responsible for the Stachyflin-resistant phenotype. Stachyflin-resistant A/WSN/33 virus clones were also resistant in virus-mediated RBC hemolysis even with a high dose of Stachyflin (Table 1). In addition, the incapability of Stachyflin to inhibit low-pH-induced conformational change of R2 or R3-type Stachyflin-resistant HA was also confirmed directly by examining the susceptibility of HA protein to trypsin digestion in the presence of Stachyflin (data not shown). These data confirmed the previously published mechanism of action of Stachyflin as a specific fusion inhibitor (27).

Most mutant viruses resistant against HA conformational change inhibitors have been reported to have HAs with an elevated optimum pH for RBC hemolysis, membrane fusion or low-pH-induced HA conformational change (4, 6, 12, 17). Stachyflin-resistant A/WSN/33 virus clones also contained amino acid substitution K51R, and this amino acid substitution has been reported to affect optimum pH for HA conformational change with A/WSN/33 virus (12). However, in our study, there was no difference in optimum pH for RBC hemolysis between Stachyflin-resistant A/WSN/33 virus clones and the wild-type A/WSN/33 virus (Table 1). Recently, Plotch et al (16) referred to the specific amino acid sub-

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Table 1. Biological characterization of Stachyflin-resistant viruses isolated in vitro

<table>
<thead>
<tr>
<th>No. of viruses isolated</th>
<th>IC₅₀ (µM)</th>
<th>ΔpH*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT assay</td>
<td>Hemolysis assay</td>
</tr>
<tr>
<td>Wild-type</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>R1</td>
<td>2</td>
<td>N.D.</td>
</tr>
<tr>
<td>R2</td>
<td>4</td>
<td>&gt;52</td>
</tr>
<tr>
<td>R3</td>
<td>2</td>
<td>&gt;52</td>
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<tr>
<td>R4</td>
<td>3</td>
<td>&gt;52</td>
</tr>
<tr>
<td>R5</td>
<td>2</td>
<td>&gt;52</td>
</tr>
<tr>
<td>R6</td>
<td>1</td>
<td>&gt;52</td>
</tr>
</tbody>
</table>

* The pH at which 50% of hemolysis induction by the wild-type virus is 4.9.

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Table 2. Summary of the amino acid substitutions in HA of Stachyflin-resistant variants isolated in vitro

<table>
<thead>
<tr>
<th>Amino acid position</th>
<th>HA1 subunit</th>
<th>HA2 subunit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td>R1</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R2</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R3</td>
<td>Ile</td>
<td>Arg</td>
</tr>
<tr>
<td>R4</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R5</td>
<td>Ile</td>
<td>Arg</td>
</tr>
<tr>
<td>R6</td>
<td>Ile</td>
<td>Arg</td>
</tr>
</tbody>
</table>

* H3 subtype numbering.

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<tbody>
<tr>
<td>Val</td>
<td>Lys</td>
<td>Lys</td>
</tr>
<tr>
<td>R1</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R2</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R3</td>
<td>Ile</td>
<td>Arg</td>
</tr>
<tr>
<td>R4</td>
<td>Arg</td>
<td>Glu</td>
</tr>
<tr>
<td>R5</td>
<td>Ile</td>
<td>Arg</td>
</tr>
<tr>
<td>R6</td>
<td>Ile</td>
<td>Arg</td>
</tr>
</tbody>
</table>

* H3 subtype numbering.

# Same amino acid as wild-type influenza A/WSN/33 virus.
Fig. 2. Effect of Stachyflin on the membrane fusion activity of WSN WT-HA or mutant HAs. Two-hundred-and-ninety-three EBNA 1 monolayers transfected with expression plasmids containing WT-HA (A) or mutant HAs (B), were briefly treated with 10 U/ml neuraminidase and 4 µg/ml trypsin for 1 hr at 37°C before the addition of R-RBC. The membrane fusion was induced with low-pH buffer in the presence (right) or absence (left) of 10 µg/ml Stachyflin. The cells were observed under laser microscopy and photographed (× 400).
stitution in HA protein as either increasing or decreasing the pH optimum for HA conformational change dependent on the virus strains examined. Considering the fact that the HA protein of the A/WSN/33 virus strain used in our experiment had several amino acid substitutions in the HA1 and HA2 subunits as compared to that of original A/WSN/33 virus and that used by others (5, 12), the discrepancy of our results with those of previous studies may not be surprising (12). The point is that Stachyflin resistance is not associated with the elevated optimum pH for membrane fusion.

The results of in vitro membrane fusion analysis revealed that amino acid substitutions K51R or K121E in the HA2 subunit were critical for the Stachyflin-resistant phenotype of A/WSN/33 HA protein (Fig. 2). These amino acids, K51 and K121, were located at helices A and D, respectively, in the HA2 subunit in the neutral pH HA conformation (3, 23). From the X-ray crystallographic analyses of the HA protein of H3 subtype influenza virus strain X31, the locations of these amino acids are dramatically rearranged under low-pH conditions (2). In addition, both amino acids are conserved at least in all HA proteins of human influenza viruses (10, 15). Therefore, they might be important in low-pH-induced HA conformational change. As for the other two amino acid substitutions, V1761 and S206L, located near the receptor binding pocket in the HA1 subunit and transmembrane region of the HA2 subunit, respectively, we could not detect any role in the in vitro HA-mediated membrane fusion assay (Fig. 2). These amino acid substitutions could happen by chance during in vitro passages independent of the selection pressure by Stachyflin.

Alternatively, for the mutant virus with amino acid substitution K51R, which we could not amplify in MDCK cells, another amino acid substitution such as V1761 or S206L might be required for efficient replication.

As previously described, the inactivation of infectivity of influenza virus by Stachyflin did not disappear by dilution or washing of the virus (27). This phenomenon suggests that Stachyflin might form a strong interaction with the specific amino acids of HA protein in neutral pH conformation, and we have attempted to construct a model which explains the characteristic properties of HA conformational change inhibition with Stachyflin (10, 20). We have obtained the crystal structure of 11-O-acetyl Stachyflin, which contained one molecule of H2O in the 2.92 Å distance from the oxygen of 2-carbonyl (data not shown). Our assumption for this docking study was that conformation of the Stachyflin molecule should be almost the same as that of 11-O-acetyl Stachyflin, and it should also contain one molecule of H2O. Another assumption was that the conformation of the HA of A/WSN/33 virus is essentially the same as that of the H3 subtype (12). However, we noticed that serine at position 113 of HA2 in the H3 subtype, H2 subtype or most of the H1 subtype is in the hydrogen bonding distance with leucine at position 2 of HA2. But the substitution of serine at position 113 to leucine in A/WSN/33 HA cancels this hydrogen bonding, and the N-terminal region of HA2 of A/WSN/33 should be flexible compared to that of the H3 subtype. It has recently been proposed that there exists a pocket among the H1 subtype HA trimer near the NH2-terminus of the HA2 subunit, in which a single, small molecule could be docked (1, 6, 12, 16). First, we have attempted to place Stachyflin into the proposed crevice in the region of the NH2-terminus formed among the HA trimer (Fig. 4). Considering the molecular size and structural rigidity of Stachyflin (Fig. 3), Stachyflin could not be docked into the proposed crevice among the HA trimer (Fig. 4a). Instead, we have found the space in which a single Stachyflin molecule can be placed in a single WSN-HA molecule (Fig. 4b). In this model, 11-O of Stachyflin could form a hydrogen bond with the N atom of lysine at position 51 which locates in helix A. Water may locate in the hydrogen bonding distance, both with the 2-carbonyl oxygen of Stachyflin and the N atom of lysine at position 121 in helix D. Thus, helices A and D may be stabilized at neutral pH conformation by Stachyflin. Amino acid substitution K51R or K121E would result in losing one of these hydrogen bondings. Next, we examined the possible binding of Stachyflin to the HA of A/Sendai/808/91 (H1N1) virus, which has serine in HA2 at position 113 (Fig. 4c). It is unlikely that Stachyflin interacts with the HA protein in the same way as when simulated with WSN-HA. However, it was expected that Stachyflin should interact with the HA protein with phenylalanine at position 110 in helix D by pi-stacking with the isoindolinone of Stachyflin and lysine at position 51 by hydrogen bonding. Interaction...
Fig. 4. Possible interaction of Stachyflin with simulated HA protein. a) Stereo view of a part of the HA2 domain based on H3 subtype HA. HA2 trimer conformation of the N-terminal domain (G1-N12) and helix D (A96-T122) is shown in cyan, magenta and red. Three-dimensional data was 1hdg. pdb, where 4 amino acids (R106, F110, N114, N117) were substituted for original H3 type acids (23). To visualize the inner size of the HA2 trimer, a triangle connecting the Cα carbons of the F110 position is drawn in yellow indicating the size of 14.0 Å, while at the position of R106, it is 10.6 Å. Molecular size of Stachyflin bound with water is about 12 × 6 Å. The cavity formed by HA2 trimer around F110 and R106 is too small to bind Stachyflin. Two oxygen atoms of the side chain of S113 (helix D) and carbonyl oxygen in the main chain of 2L (N-terminal fusogenic region) are shown in pink. They make hydrogen bonding in the 808 strain, whereas in the WSN strain, S113 is replaced by L113, which may lead to the N-terminal region being more flexible. b) A modeled interaction of Stachyflin with the HA2 region in the WSN strain. To clarify, only two subunits of HA2 trimer are shown in cyan and red. Amino acids A36-I56 in helices A and A* and A96-T122 in helices D and D* are depicted. The Cα position of G47 is marked by a purple circle. Stachyflin bound with water is docked between helices A and D of another unit of HA2 so that there are the two hydrogen bonding interactions between the amino N atom of K61 side chain and carbonyl oxygen of Stachyflin, and also between the amino N atom of K121 side chain and a water molecule bound to Stachyflin. This interaction is made possible by removing the N-terminal portion as well as the sterical allowance due to G47. c) A modeled interaction of Stachyflin with the HA2 region in the 808 strain. Two sets of helices A and D are shown in cyan and red, the same as Fig. 4b. The main interaction is assumed to be pi-stacking between benzene ring F110 and the isoindoline moiety of Stachyflin, here also, G47 made the rotation of F110 possible. Q105 (see Fig. 4a) may also play van der Waals interaction with Stachyflin. The stereo drawings of Fig. 4, a–c were prepared using MolMol (11).
with glutamine at position 47 of the HA2 subunit because of steric hindrance by the side chain of glutamine.

The molecular mechanism of Stachyflin interaction with the HA protein we have proposed in this paper is remarkable for the following point; that is, three molecules of Stachyflin should interact with a trimer HA complex. Obviously, there is a need to perform an X-ray crystallographic analysis of Stachyflin complexed with HA subtypes to further understand the molecular mechanism of action of Stachyflin.

References


nuclear magnetic resonance spectroscopy and X-ray crystallography. Biochemistry 31: 9609.


