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Site-Specific Mutational Analysis of a Novel Cysteine Motif Proposed To Ligate the 4Fe-4S Cluster in the Iron-Sulfur Flavoprotein of the Thermophilic Methanoarchaeon *Methanosarcina thermophila*

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Isf (iron-sulfur flavoprotein) from *Methanosarcina thermophila* has been produced in *Escherichia coli* as a dimer containing two 4Fe-4S clusters and two FMN (flavin mononucleotide) cofactors. The deduced sequence of Isf contains six cysteines (Cys 16, Cys 47, Cys 50, Cys 53, Cys 59, and Cys 180), four of which (Cys 47, Cys 50, Cys 53, and Cys 59) comprise a motif with high identity to a motif ($CX_2CX_2CX_{4-7}C$) present in all homologous Isf sequences available in the databases. The spacing of the motif is highly compact and atypical of motifs coordinating known 4Fe-4S clusters; therefore, all six cysteines in Isf from *M. thermophila* were altered to either alanine or serine to obtain corroborating biochemical evidence that the motif coordinates the 4Fe-4S cluster and to further characterize properties of the cluster dependent on ligation. All except the C16S variant were produced in inclusion bodies and were void of iron-sulfur clusters and FMN. Reconstitution of the iron-sulfur cluster and FMN was attempted for each variant. The UV-visible spectra of all reconstituted variants indicated the presence of iron-sulfur clusters and FMN. The reduced C16A/S variants showed the same electron paramagnetic resonance (EPR) spectra as wild-type Isf, whereas the reduced C180A/S variants showed EPR spectra identical to those of one of the two 4Fe-4S species present in the wild-type Isf spectrum. Conversely, EPR spectra of the oxidized C50A and C59A variants showed *g* values characteristic of a 3Fe-4S cluster. The spectra of the C47A and C53A variants indicated a 4Fe-4S cluster with *g* values and linewidths different from those for the wild type. The combined results of this study support a role for the novel $CX_2CX_2CX_{4-7}C$ motif in ligating the 4Fe-4S clusters in Isf and Isf homologues.

Two-thirds of the biologically produced methane in nature originates from the methyl group of acetate in a pathway where acetate is cleaved and the methyl group is reduced to methane with electrons derived from oxidation of the carbonyl group to carbon dioxide (7). Much is known concerning the cleavage of acetate and one-carbon transfer reactions; however, less is known regarding electron transport. Recently, a novel iron-sulfur flavoprotein (Isf) from the acetate-utilizing methanoarchaeon *Methanosarcina thermophila* was characterized (3, 12). The homodimeric Isf contains two flavin mononucleotide (FMN) molecules and two 4Fe-4S clusters unequivocally identified by electron paramagnetic resonance (EPR) and Mössbauer spectroscopy. The midpoint potential values of the 4Fe-4S cluster and FMN are −394 and −277 mV, respectively. These results are the basis for a postulated role for the cluster in electron flow from ferredoxin A, the physiological electron donor for Isf, to the 4Fe-4S cluster and then to the FMN of Isf. The physiological electron acceptor for Isf is unknown. The deduced sequence of Isf contains six cysteines, four of which are in an unusually compact novel motif with high identity to a motif ($CX_2CX_2CX_{4-7}C$) that is conserved among all homologous Isf sequences identified in the databases. This observation suggests that the motif ligates the 4Fe-4S clusters in Isf; however, corroborating biochemical evidence has not been reported.

The cubane 4Fe-4S cluster is ubiquitous in proteins from all domains of life, where it mainly functions in electron transfer (17). The sulfur atom of cysteine is the prominent protein ligand coordinated to iron atoms in these clusters. The coordination of 4Fe-4S clusters by amino acids other than cysteine is rather uncommon. Examples of variations from cysteine ligation include aconitase with oxygen ligation originating from hydroxide, water, or substrate. The 4Fe-4S cluster in the ferredoxin from *Pyrococcus furiosus* is ligated with oxygen from aspartate (21). An iron atom in the 4Fe-4S cluster of hydrogenase from *Desulfovibrio gigas* is coordinated by a histidyl nitrogen (20). A single motif (CX_2CX_2C plus a distal C in the polypeptide chain) coordinates all low-potential, redox-active 4Fe-4S clusters for which cysteine is the exclusive ligand. Possible exceptions to this ubiquitous 4Fe-4S motif are found in the corrinoid/iron sulfur proteins of *M. thermophila* and *Clostridium thermoaceticum*, and a putative iron-sulfur protein from *Rhodobacter capsulatus*, where the sequence $CX_2CX_4CX_{16}C$ is perfectly conserved (13); however, investigations of involvement of this motif in ligation of 4Fe-4S clusters have not been reported. Thus, the highly conserved $CX_2CX_2CX_{4-7}C$ motif in Isf is the most compact motif known with the potential to coordinate a 4Fe-4S center. Although the great majority of iron-sulfur proteins function in electron transfer reactions, the clusters in a few proteins function in nonredox catalysis or serve a structural role. Still other iron-sulfur clusters bind nucleic acids or play a regulatory role (4, 10). Two of these, endonuclease III and MutY, contain a redox-inert 4Fe-4S clus-

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ter coordinated by a compact cysteine motif ($CX_6CX_2CX_5C$) (15, 16).

Using site-specific replacement of residues ligating the clusters (14), much has been learned regarding the coordination of iron-sulfur clusters, particularly the identities of ligating residues. Changes in spectroscopic properties and other characteristics have provided information regarding the polypeptide environment of the cluster and the effects that the coordinating ligands have on the biochemical properties of the cluster. Thus, a series of site-specific replacements in Isf from *M. thermophila* were performed to obtain corroborating biochemical evidence for the proposed role of the novel cysteine motif and further characterize the properties of the 4Fe-4S cluster dependent on ligation. The results support involvement of the motif in coordination of the 4Fe-4S cluster.

MATERIALS AND METHODS

Sequence comparisons. Microbial genomic sequence databases were searched online (http://www.tigr.org). Sequences were aligned using the program Clustal X, version 1.64b.

Plasmid construction and site-directed mutagenesis. Plasmid pML701, which contains the entire gene for Isf, was used as a template to construct mutants. Site-directed mutagenesis was performed using MORPH as described by the manufacturer (5 Prime → 3 Prime, Inc., Boulder, Colo.). Each construct was confirmed for the intended mutation by sequencing using the automated dideoxy method at the Pennsylvania State University nucleic acid facility.

Protein production and purification. *Escherichia coli* BL21(DE3) cells transformed with derivative expression plasmids carrying the designated *isf* mutations were grown on Luria-Bertani broth supplemented with ampicillin (100 μg/ml). Once cells reached an A_{600} of about 0.8, they were induced to produce high levels of the Isf variants by addition of 1% (wt/vol [final concentration]) Bacto-Lactose for 2 h. The cells were harvested by centrifugation at 11,800 × *g* for 10 min at 4°C. The cell pellets were frozen at −70°C.

The C16S variant and wild type were purified as described elsewhere (12). All other variants were purified as follows. Approximately 5 g (wet weight) of cells was suspended in 6 volumes (wt/vol) of buffer A (50 mM Tris-HCl [pH 7.6], 200 μg of lysozyme/ml, 2 mM dithiothreitol [DTT]) and incubated for 20 min at 21°C. Cells were lysed by two passages through a French pressure cell at 20,000 lb/in^2. The lysate was centrifuged at 10,000 × *g* for 30 min at 4°C. The pellet, containing inclusion bodies, was washed twice in 30 ml of buffer B (50 mM Tris-HCl [pH 7.6], 2 M urea, 1% Triton X-100, 2 mM DTT). The protein aggregates were solubilized in 2 ml of buffer C (50 mM Tris-HCl [pH 7.6], 6 M guanidine-HCl) and incubated for 2 h at 21°C. Insoluble protein was removed by centrifugation at 10,000 × *g* for 10 min at 4°C. The protein solution at this stage is termed denatured. The soluble fraction was then diluted 100-fold in buffer D (50 mM Tris-HCl [pH 7.6], 500 mM L-arginine, 2 mM DTT) and incubated at 4°C for 12 h. In the following step, the sample was concentrated using polyethylene glycol 8000. The protein was dialyzed in buffer E (50 mM Tris-HCl [pH 7.6], 250 mM L-arginine, 200 mM NaCl, 2 mM DTT) and then buffer F (50 mM Tris-HCl [pH 7.6], 200 mM NaCl, 2 mM DTT). The protein at this stage is defined as renatured. There was no apparent change in subunit size among the wild type and variants as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The overall procedure resulted in homogeneous proteins as judged by SDS-PAGE.

Reconstitution of iron-sulfur clusters and FMN into renatured apoprotein. Reconstitution of iron-sulfur clusters and FMN was performed by adding 1 ml of 10 mM FMN, 800 μl of 2-mercaptoethanol, 300 μl of 60 mM $FeCl_3$, and 300 μl of 60 mM Na_2S to 100 ml of renatured apoprotein solution (2, 11). All reagents were added dropwise with 10-min intervals between steps, and the reconstitution reaction mixture was incubated at 4°C for 12 h. This procedure is identical to the one used for reconstitution of iron-sulfur clusters into the PsaC subunit of photosystem I (2, 11). The protein was concentrated with an ultrafiltration unit fitted with a YM 30 membrane (Amicon, Beverly, Mass.), and the unbound molecules were removed by a PD10 gel filtration. The protein at this step is called reconstituted. Iron and FMN were determined as previously described (12). Reduction with ferredoxin A was as described elsewhere (12).

Spectroscopy. UV-visible spectra were obtained with a Hewlett-Packard 8452A diode array spectrophotometer. EPR signals of iron sulfur clusters were recorded with a Bruker ECS 106 EPR X-band spectrometer operating with an ER/4102 ST or 4116 DM resonator and an Oxford liquid helium cryostat (Oxford Instruments, Oxford, United Kingdom). The temperature was controlled using an ITC4 Oxford temperature controller. The microwave frequency was determined with a Hewlett-Packard 5340A frequency counter. The spectrometer conditions are described in the figure legends and Results. The term oxidized is used to denote proteins as they were purified and without the addition of sodium dithionite. To detect the EPR signal of $[4Fe\text{-}4S]^{1+}$ clusters, the sample was reduced by the addition of 0.1 ml of 1.0 M pH 10 glycine buffer to 0.2 ml of sample, thus changing the pH of the sample to 10, followed by the addition of approximately 1 mg of sodium dithionite. It was necessary to increase the final pH of the sample to 10.0, thereby lowering the solution potential to ensure full reduction of low potential iron-sulfur clusters (2). This standard procedure is identical to that previously described for analysis of PsaC (2). Binary EPR data were processed with a macro command program written by I. Vassiliev using Igor Pro, version 3.14 (Wavemetrics), as described elsewhere (19). EPR spectra were recorded at different times using several different X-band resonators, which operate on slightly different microwave frequencies. This resulted in small differences in the positions of identical signals when plotted against the magnetic field axis. Therefore, we plotted spectra against the *g*-value axes by converting experimental data points recorded against magnetic field reference values using the formula $g = 714.484 \cdot f/H$, where f is microwave frequency in megahertz and H is magnetic field in Gauss. This procedure allowed the direct comparison of EPR spectra on the same scale. The top axes of the EPR spectra show the magnetic field scales, which were calculated back from the *g*-value scale by employing the same formula and a frequency of 9.4676 MHz (4102 EPR cavity) (11). This procedure allowed plotting signals using identical *g*-value and magnetic field scales, which allowed for a direct comparison of the signals. Apparent *g* values are used for the description of EPR data.

RESULTS

Sequence comparisons of Isf from *M. thermophila* with Isf homologues. Figure 1 shows that metabolically diverse species contain open reading frames with deduced sequence identity to *M. thermophila* Isf, suggesting that it functions in carbon dioxide-reducing (*Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*) and sulfate-reducing (*Archaeoglobus fulgidus*) members of the domain *Archaea*, and also in metabolically diverse procaryotes from the domain *Bacteria* (*Chlorobium vibrioforme*, *Chlorobium tepidum*, and *Clostridium difficile*). Comparison of these sequences with Isf from *M. thermophila* shows an unusually compact N-terminal cysteine motif with a strictly conserved spacing of $CX_2CX_2CX_{4-7}C$, which is atypical of the cysteine motif coordinating all known redox-active 4Fe-4S centers. The absence of motifs known to ligate redox-active 4Fe-4S clusters and conservation of this novel motif among Isf sequences suggest involvement in ligation of the 4Fe-4S clusters in Isf; thus, we undertook a biochemical approach to obtain corroborating experimental evidence for the proposed role of the motif in the Isf from *M. thermophila* and further investigate properties of the 4Fe-4S cluster dependent on ligation.

Heterologous production, purification, and reconstitution of wild-type Isf and variants. All six cysteines present in Isf (Fig. 1) were individually altered to either alanine or serine. When produced in *E. coli*, all variants except C16S were contained in inclusion bodies. The soluble C16S was purified the same as for the wild type. The presence in inclusion bodies is indicative of protein misfolding, a characteristic of variant iron-sulfur proteins in which the cluster is absent or inserted incorrectly (14). Indeed, no iron-sulfur clusters were detected by EPR spectroscopy for any of the isolated inclusion bodies containing variants. These results are consistent with the proposal that the conserved cysteine motif comprised of Cys 47, Cys 50, Cys 53, and Cys 59 ligates the 4Fe-4S cluster in Isf from *M. thermophila*. However, it is also possible that any of the variants misfolded as a consequence of factors unrelated to the inability to incorporate the 4Fe-4S cluster; thus, reconstitution of an iron-sulfur cluster and FMN was attempted. The isolated inclusion bodies were extracted in guanidine hydrochloride, which solubilized the proteins. At this juncture, no discrete bands were detected by native PAGE, suggesting that the proteins were denatured. The solubilized variants were diluted in buffer containing arginine, which was necessary to prevent protein aggregation during renaturation (1, 6, 18). After removal of the arginine by dialysis, native PAGE indicated no discrete bands, suggesting that the proteins had not achieved the native state. Iron-sulfur clusters and FMN were not de-

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                                  16                                         47
MST     ---------M KITGISGSPR KGQNCEKIIG AALEVAKERG FETDTVFISN EEVAP--CKA   49
MCJ-2   ---------M KVIGISGSPR PEGNTTLLVR EALNAIAEEG IETEFISLAD KELNP--CIG   49
MBT-1   MKQKEVDFMV KVIGICGSPR KNGNTEILLR EALDAAEEAG AETELVRLAG LDINP--CRA   58
MBT-2   ---------- MILGICGSPR K-QATEHVLE RALSMLEDDG LETEFFTVRG KNISP--CRH   47
AF-2    ---------- MIVGISGSPR R-KATEFVLG EALKMLEERG FETKFFTVRG KKISP--CQH   47
MCJ-1   ---------M KVFGISGSPR L-QGTHFAVN YALNYLKEKG AEVRYFSVSR KKINF--CLH   48
CV      ---------M KVIGINGSPR PAGNTSIMLK TVFETLEQEG IETELIQVGG TDIKG--CRA   49
CT      ---------M KVIGINGSPR RAGNTSIMLK TIFEVLEDEG IETELIQVGG TNIKG--CRA   49
AF-3    ---------M KLLAINGSPN K-RNTLFLLE VIAEEVKKLG HEAEIIHLKD YEIKE--CKG   48
MBT-3   ---------- ---------- ------MVLE HCRDAIESHG VETDIISLRG MKIES--CRA   32
AF-1    ---------M KAVGILGSPR KYGNASKMLD AALKELENSG FEVEKVHISS KKINY--CTG   49
CD      ---------M IITVMNGSPR KNGATSKVLT YLYKDIERLI PDVKINYFDL SEVNPSYCIG   51

        50 53      59
MST     CGACRDQDF- -CVID-DDMD EIYEKMRAAD GIIVAAPVYM GNYPAQLKAL FDRSVLLRR-  105
MCJ-2   CNMCKEEGK- -CPII-DDVD EILKKMKEAD GIILGSPVYF GGVSAQLKML MDRSRPLR--  104
MBT-1   CDSCKKTGE- -CAIE-DDLN RVVELAASAH GIIIGSPVYF GSVTAQTKMF MDRTRPLR--  113
MBT-2   CDYCLRNKE- -CVLK-DDMF PLYELLRRAA GIIIATPVYN GGVSAQIKAI MDRCRALGAE  104
AF-2    CDYCLKHKE- -CRIK-DDMF ELYEMLKDAK GIVMATPVYN GGVSAQIKAV MDRCRALVAA  104
MCJ-1   CDYCIKKKEG -CIHK-DDME EVYENLIWAD GVIIGTPVYQ GNVTGQLKTL MDRCRAILAK  106
CV      CYACIRNKNS KCSTK-DGFN EIFEKMVEAN GMILGSPVYF ADITPELKAL IDRSGFVSRT  108
CT      CYACIKNKNS ECSTKGDGFN EIFAKMVEAD GMILGSPTYF ADITPELKAL IDRAGFVSRT  109
AF-3    CDACLKGD-- -CSQK-DDIY KVLEKMQEAD AIVIGTPTYF GNVTGIVKNL IDRSRMAR-M  103
MBT-3   CLSCAKKHR- -CRID-DGLN DIIDRIRDSE GFIVATPVYF GTARGDLMAA LQRIGMVSRA   89
AF-1    CGTCLAKGE- -CVQR-DDMD ELKRLVEESD AVILASPVYY LNVTAQMKTF IDRMLPYG--  104
CD      CLNCYKMGK- -CINQNDKVE YIHDIITKSD GVIFGSPTYG SSVTGLFKVF TDRAHMML--  107

MST     KNFALKNKVG AALSVGGSRN GGQEKTIQSI HDWMHIHGMI VVGDNS---- HFGGI---TW  158
MCJ-2   IGFQLRNKVG GAVAVGASRN GGQETTIQQI HNFFLIHSMI VVGDND-PTA HYGGT---GV  160
MBT-1   SEFRLANRVG GAVTVGGSRN GGQETACRDI HSFFLIHEAA VVGNAS-PTA HYGGT---GV  169
MBT-2   DYDSLRGKVG MGIAVGGDRC GGQEPALMQI HTFYILNGVI PVSGGS-FGA NLGAC---FW  160
AF-2    DYDFFRGKVG MAIAVGGDRI GGQELAIQQI LTFYILNGVI PVSGGS-FGA NIGAT---FW  160
MCJ-1   NPKVLRGRVG MAIAVGGDRN GGQEIALRTI HDFFIINEMI PVGGGS-FGA NLGAT---FW  162
CV      NGQLFRHKVG ASIVS--LRR GGGVHAYDSI NHLFQICQMF MVGSTY---W NLG-----FG  158
CT      NGQLFRHKVG ASVVS--LRR GGGIHAYDSI NHLFQICQMF MVGSTY---W NLG-----FG  159
AF-3    GNYRLRNRVF APVVTSGLRN GGAEYAAMSL IVYALGQAML PVSIVE-NPI TTGTFPVGVI  162
MBT-3   SDGFLSWKVG GPIAV--ARR GGHTATIQEL LMFYFINDMI VPGSTY-WNM VFG-------  139
AF-1    HRPTLKGKYG GSIVVY-AGV GKPEEVAGYM NRVLKAWGIV PVGYAVGFGV IPGEVGDEDL  163
CD      ERLLYRKPCI AVTTY--ENA RGS-KAISFI KSMVLDSGGY VCGSLS---I KTG------F  155

                                       180
MST     NPAE------ EDTVGMQTVS E-TAK--KLC D-----VLEL IQKNR----- -------DK- -  191
MCJ-2   GKAP------ GDCKNDDIGL E-TAR--NLG K-----KVAE VVKLI----- -------KK- -- 193
MBT-1   GGAK------ GESADDMTGI E-TAR--NLG R-----RVAL LAARI----- -------HG- -- 202
MBT-2   SRDT-L---- EVLKRTHMDS KPSKRPWACL KGSWTLKDPE ILFYS----- -------EFI -- 203
AF-2    SRDT-L---- EGVKEDEEGF R-SLR--KTV K-----RFAE MLEKM----- -------EGV -- 195
MCJ-1   SKDRGK---- KGVEEDEEGL R-VLR--KTL N-----RFYE VLKEK----- -------RGL -- 198
CV      GRDG------ GEVVNDTEGM D-NMR--DLG K-----SMAF LLKKL----- -------NAS -- 192
CT      GRDG------ GEVVNDTEGM E-NMR--DLG H-----SMAF LLK------- ---------- -- 188
AF-3    QGDAGW---- RSVKKDEIAI N-SAK--ALA KR--IVEVAE ATKNL----- -------RES -- 201
MBT-3   -WAP------ GEVEDDSEGI E-TIR--RFG E-----NVAE LIKRI----- -------NGG S- 173
AF-1    KKASQLGSKI AEAFESKYRM EPSDEDLELQ K-----QLLT LIKNYGHLMK ADYEFWKEKG FI 220
CD      NQNP------ ---------- ---------- ---------- ---------- ---------- -- 159
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FIG. 1. Multiple amino acid sequence alignment of Isf from *M. thermophila* (MST) with sequences deduced from open reading frames identified in the genomic sequences of *Methanococcus jannaschii* (MCJ), *Methanobacterium thermoautotrophicum* (MBT), *Archaeoglobus fulgidus* (AF), *Chlorobium vibrioforme* (CV), *Chlorobium tepidum* (CT), and *Clostridium difficile* (CD). Numbers after the abbreviated organism names indicate the different protein isoforms. Database codes for proteins: MST, GenBank U50189; MCJ-1 and -2, GenBank C64391 and B64435; MBT-1, -2, and -3, GenBank AE000802, AE000908, and AE000919; AF-1, -2, and -3, GenBank AE0010041, AE0009971, and AE0009721; CV, EMBL Z83933.1; CT, C tepidum gct10; CD, CD shotgun.dbs cd2h6.q1t. Cysteines (at positions 16, 47, 50, 53, 59, and 180) in Isf of *M. thermophila* are numbered above the top line. Residues conserved in at least 7 out of 10 sequences are shaded in gray. Putative FMN binding regions in Isf are underlined.

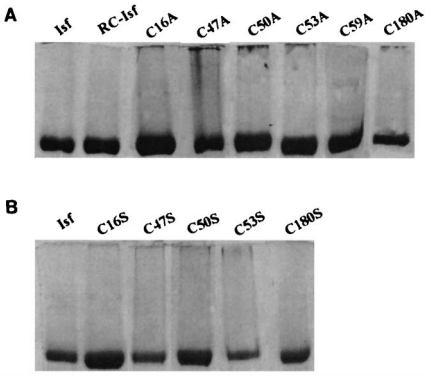

FIG. 2. Native PAGE of wild-type Isf and variants. (A) As-purified wild type (Isf), reconstituted wild type (RC-Isf), and alanine variants. Each lane was loaded with 25 μg of protein except for C180A, which was loaded with 18 μg. (B) As-purified wild type (Isf) and serine variants. The Isf, C47S, C53S, and C180S lanes were loaded with 18 μg of protein; all other lanes were loaded with 25 μg of protein.

tected by UV-visible spectroscopy; thus, the apoproteins were incubated in the presence of ferric iron, sulfide, 2-mercaptoethanol, and FMN in an attempt to reconstitute the redox centers. Native PAGE (Fig. 2) indicated a discrete band for each variant migrating to approximately the same position as the purified wild type, a result which suggested that all were in the native conformation and dimeric, in accord with the wild type (12). UV-visible spectroscopy (Fig. 3 and 4) indicated incorporation of FMN and iron-sulfur clusters. Neither FMN nor iron-sulfur clusters could be reconstituted separately, suggesting that both are required for proper folding of the protein. There was no apparent change in subunit size among wild-type Isf and variants as judged by SDS-PAGE (data not shown). There was intermittent success and low yields in the reconstitution of C59A/S and C180A/S, indicating that these variants were unstable. The identical denaturation/renaturation/reconstitution process was performed for the wild type. As for the variants, only the reconstituted wild type exhibited a discrete band after native PAGE (Fig. 2). These results suggested overall structural integrity of the C16A/S, C47A/S, C50A/S, and C53A/S variants; thus, the presence in inclusion bodies is likely the result of the inability to incorporate the 4Fe-4S cluster or FMN, or both.

Characterization of reconstituted wild-type Isf and variants. The UV-visible spectra of denatured and renatured wild-type Isf showed no absorbance characteristic of either iron-sulfur clusters or FMN (Fig. 5), suggesting the complete loss of both redox components. The UV-visible spectrum of the reconstituted wild-type Isf was nearly identical to that of the as-purified wild type, suggesting that the properties of both proteins were similar. However, the intensity of absorbance between 350 and 500 nm was threefold less for reconstituted than for as-purified Isf, suggesting incomplete incorporation of FMN and iron-sulfur centers. This trend applied to all of the reconstituted variant proteins. Both iron and FMN were present in reconstituted wild type, C16A/S, C47A/S, C50A/S, and C53A/S. The contents ranged from 3.7 to 7.2 iron atoms/dimer and from 0.5 to 1.7 molecules of FMN/dimer; however, the content of iron and FMN was variable between preparations, precluding accurate comparisons among the reconstituted variants and wild type. The iron and FMN contents were not determined for the remaining variants due to low yield and instability. The UV-visible absorption spectra for the reconstituted C16A/S variants were also similar to the wild-type spectra (Fig. 3 and 4);

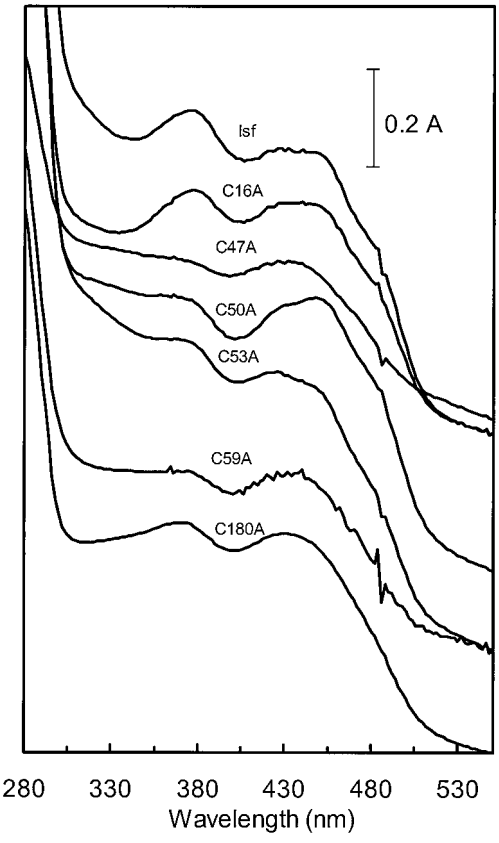

FIG. 3. UV-visible absorption spectra of wild-type Isf and alanine variants. The samples (500 μl) were in 50 mM Tris-Cl (pH 7.6) containing 200 mM NaCl. Spectra were recorded at 21°C. The amount of as-purified wild-type Isf was 80 μg; the amount of each variant was 120 μg.

however, the absorbance maxima and relative intensities in the spectra for the C47A/S, C50A/S, C53A/S, C59A, and C180A variants were a departure from spectra for reconstituted wild-type Isf and the C16A/S variants. These results suggest alterations in the properties of either or both redox centers in all except the C16A/S variants. The UV-visible spectrum of C59S contained no features characteristic of iron-sulfur cluster incorporation, a result that was confirmed by EPR spectroscopy (data not shown).

Ferredoxin A reduced the as-purified and reconstituted wild type at similar rates when normalized to the FMN content (as purified, $\Delta A_{476} = 0.52 \pm 0.03$/min/μmol of FMN; reconstituted, $\Delta A_{476} = 0.59 \pm 0.03$/min/μmol of FMN). The C16A/S, C47A/S, C50A/S, and C53A/S variants were all reduced with ferredoxin A; however, the rates were highly variable between preparations, precluding accurate comparisons. Measurements for reduction of C180A/S or C59A/S were not possible due to instability of these variants.

EPR spectroscopy of reconstituted wild-type and variants. EPR spectroscopy was performed for the wild type and variants to further characterize the reconstituted iron-sulfur centers. A summary of the results is presented in Table 1. The full power and temperature dependence profiles of the signals were recorded for all reduced resonances discussed. All resonances had temperature optima of around 15 K, and none were saturated under the following conditions: temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 G. Thus, none of the signals shown in Fig. 6 and 7 are perturbed

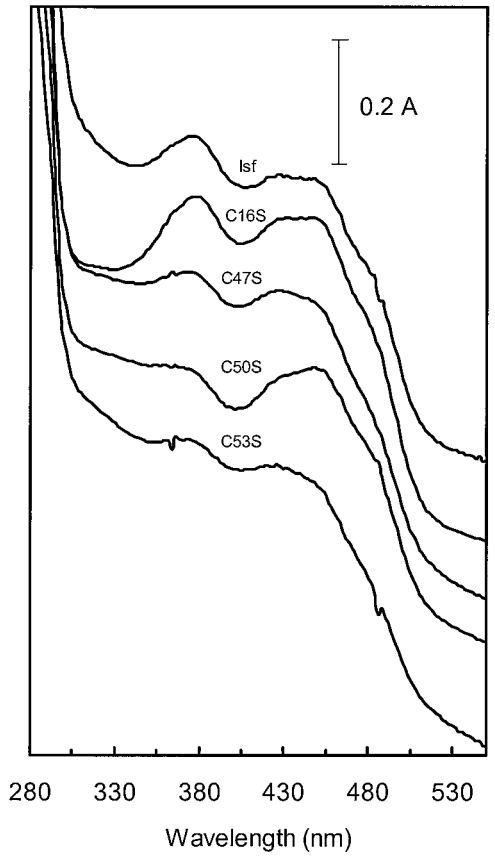

FIG. 4. UV-visible absorption spectra of wild-type Isf and serine variants. The samples (500 μl) were in 50 mM Tris-Cl (pH 7.6) containing 200 mM NaCl. Spectra were recorded at 21°C. The amount of as-purified wild-type Isf was 80 μg; the amount of each variant was 120 μg.

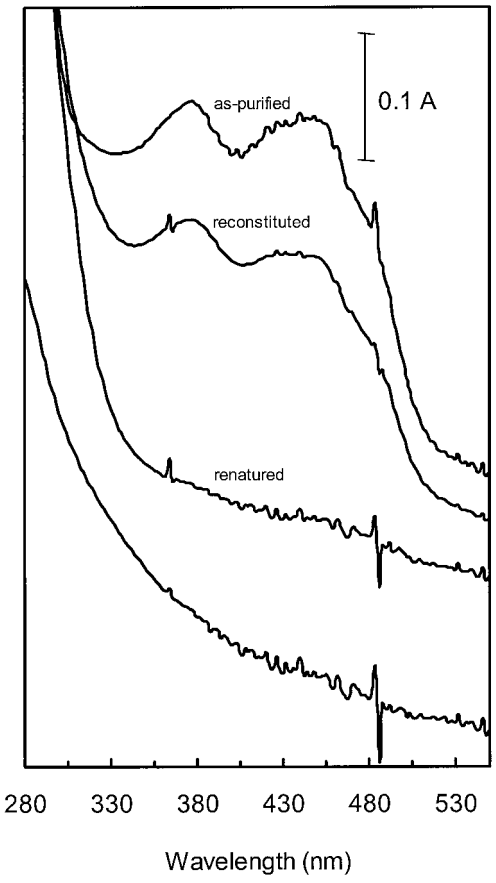

FIG. 5. UV-visible absorption spectra of as-purified, denatured, renatured, and reconstituted wild-type Isf. The denatured protein was in 50 mM Tris-Cl (pH 7.6) containing 6 M guanidine HCl. The as-purified, renatured, and reconstituted proteins were in 50 mM Tris-Cl (pH 7.6) containing 200 mM NaCl. The spectra were recorded at 21°C. The amounts used were 80 μg (as-purified wild-type Isf) and 150 μg (all others).

due to saturation. Resonances of wild-type Isf and all variants are shown under identical experimental conditions, allowing direct comparisons (Fig. 6 and 7).

The spectra recorded for reduced samples of wild-type Isf, C16A/S, C47A, C53A, and C180A/S were broadened beyond observation above 40 K, a result typical for reduced $[4Fe\text{-}4S]^{1+}$ clusters. We were unable to saturate these signals even when high microwave powers were applied to the samples, a quality also typical for $[4Fe\text{-}4S]^{1+}$ centers. These two lines of evidence rule out the possibility that the observed signals derived from 2Fe-2S centers in the wild type or any of the variants and provide strong evidence supporting the presence of 4Fe-4S centers in these proteins.

The reduced as-purified wild-type Isf exhibited a spectrum with apparent *g* values of 2.06, 2.03, 1.92, 1.86, and 1.81 (Fig. 6A), results that are nearly identical to those in a previous report (3) in which the authors attributed the complexity of the spectrum to heterogeneity of the sample. We were able to distinguish two distinct species based on power and temperature dependencies (data not shown), one with apparent *g* values of 2.06, 1.92, and 1.81 and another with apparent *g* values of 2.03, 1.92, and 1.86. The ratio of these species varied in different Isf preparations, suggesting that the as-purified wild-type protein exists in two distinct conformational states. Reconstitution of wild-type Isf that had been denatured and renatured also exhibited a $[4Fe\text{-}4S]^{1+}$ EPR spectrum with apparent *g* values and linewidths identical to those for the wild-type spectrum (Fig. 6B), with evidence for both species originally present in the wild-type as-purified Isf spectrum.

The reduced C16A/S variants exhibited EPR spectra with apparent *g* values of 2.06, 2.04, 1.92, 1.86, and 1.82 (Fig. 6C and

TABLE 1. EPR properties of wild-type Isf and variants

Protein	Apparent *g* values	Iron-sulfur center type
Wild type		
As purified	2.06, 1.92, 1.81 (species I) 2.03, 1.92, 1.86 (species II)	[4Fe-4S]
Reconstituted	2.06, 1.92, 1.81 (species I) 2.03, 1.92, 1.86 (species II)	[4Fe-4S]
Variants		
C16A/S	2.06, 1.92, 1.82 (species I) 2.04, 1.92, 1.86 (species II)	[4Fe-4S]
C47A	2.05, 1.93, 1.89	[4Fe-4S]
C50A	2.01, 1.98	[3Fe-4S]
C53A	2.03, 1.91, 1.89	[4Fe-4S]
C59A	2.02, 1.99	[3Fe-4S]
C180A/S	2.04, 1.93, 1.87; with minor contribution from 2.06, 1.93, 1.81	[4Fe-4S]

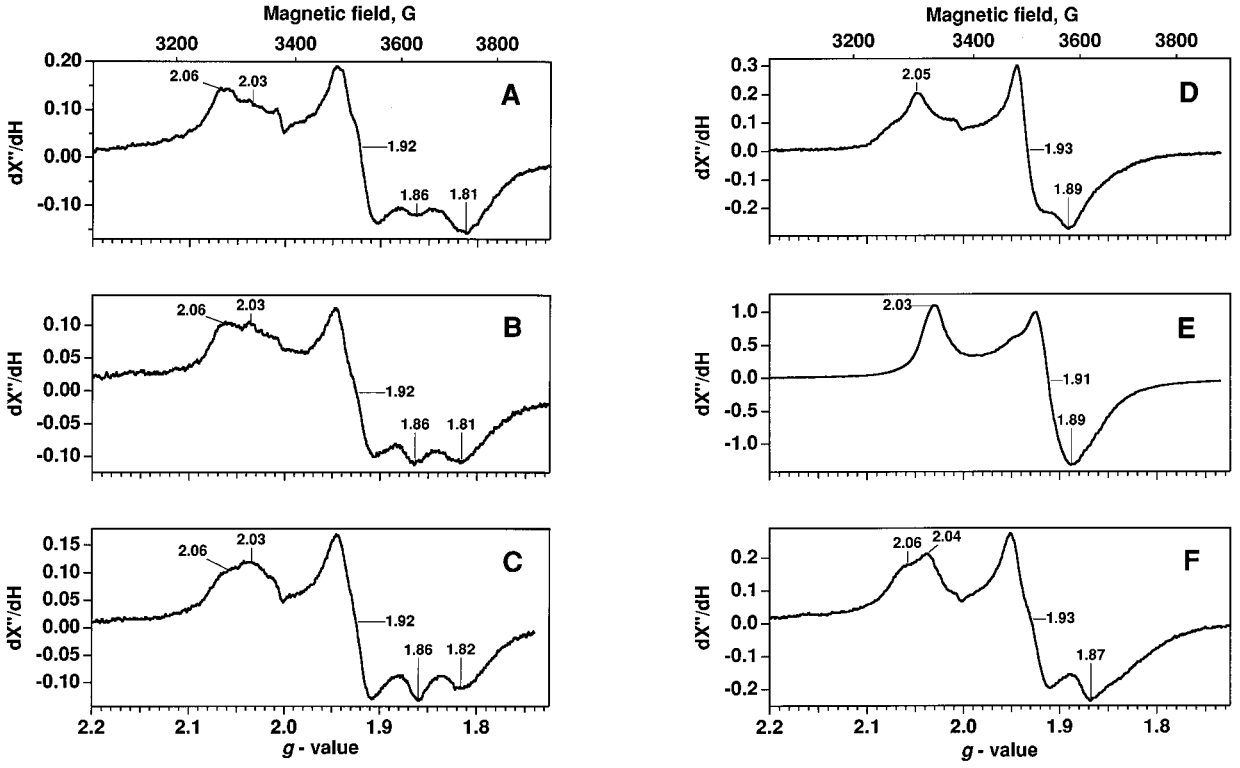

FIG. 6. EPR spectra of wild-type Isf and alanine variants. (A) Reduced as-purified wild-type Isf. (B to F) Reduced reconstituted wild-type Isf, C16A, C47A, C53A, and C180A variants. (G and H) Oxidized reconstituted C50A and C59A variants. EPR conditions: temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 G. The intensity of the EPR signal presented on trace H was multiplied by 10 for presentation purposes.

7A), identical to results for wild-type Isf with two distinct 4Fe-4S species. These results indicate that Cys 16 does not participate in ligation of the 4Fe-4S cluster of Isf, consistent with sequence comparisons showing that Cys 16 is not conserved with Isf homologues (Fig. 1). The reduced C180A/S variants showed EPR spectra (Fig. 6F and 7B) with linewidths and apparent g values (2.04, 1.93, and 1.86) nearly identical to those for one of the 4Fe-4S species present in the as-purified wild-type Isf spectrum. A minor contribution of the other species was also observed as a shoulder at $g = 2.06$.

Except for C59S, UV-visible spectroscopy of the reconstituted variants suggested that an iron-sulfur cluster was present; thus, these variants were further examined by EPR spectroscopy. Spectroscopy of the reduced C50A or C59A variants detected no $[4Fe\text{-}4S]^{1+}$ cluster; however, spectra of the oxidized variants showed a low-intensity signal with linewidths and g values typical for $[3Fe\text{-}4S]^{1+}$ clusters (Fig. 6G and H). EPR spectra of oxidized as-purified or oxidized reconstituted wild-type Isf showed no features indicating a $[3Fe\text{-}4S]^{1+}$ cluster. These results show that removal of Cys 50 and Cys 59 causes formation of the 3Fe-4S cluster in place of the 4Fe-4S cluster, a result which clearly establishes that these cysteines are involved in ligation of the 4Fe-4S cluster. A $[4Fe\text{-}4S]^{1+}$ cluster was detected in both of the reduced C47A and C53A variants by EPR spectroscopy (Fig. 6D and E); however, there were significant differences in the linewidths and g values between the spectra of the two variants. The linewidths and g values for both variants were also significantly different from the spectra of either of the two species present in the reduced form of as-purified or reconstituted wild-type Isf (compare Fig. 6A and B and Fig. 6D and E). A low-intensity $[3Fe\text{-}4S]^{1+}$ EPR signal was detected in the oxidized C47A and C53A variants (data not shown).

The EPR spectra (not shown) of the oxidized and reduced C47S, C50S, and C53S variants indicated only the presence of 4Fe-4S clusters; however, the spectral features did not contribute toward understanding the identity of the ligands that replaced cysteine.

DISCUSSION

Several lines of evidence support the view that the cysteine motif comprised of Cys 47, Cys 50, Cys 53, and Cys 59 ligates the 4Fe-4S cluster in Isf from *M. thermophila*. EPR spectroscopy strongly indicates involvement of Cys 50 and Cys 59. First, low-potential 4Fe-4S clusters are observed by EPR spectroscopy in only the reduced +1 state; thus, the inability to detect $[4Fe\text{-}4S]^{1+}$ clusters in reconstituted C50A and C59A serves as strong evidence that 4Fe-4S centers are not formed in these variants. Second, there are several examples of 4Fe-4S→3Fe-4S conversions resulting from the substitution of a ligating cysteine (14); thus, EPR evidence for $[3Fe\text{-}4S]^{1+}$ clusters in oxidized C50A and C59A adds convincingly to the evidence that Cys 50 and Cys 59 are involved in ligation of the 4Fe-4S cluster in Isf. The results also show that other ligands cannot substitute for Cys 50 and Cys 59 to preserve the 4Fe-4S cluster in these variants.

EPR results strongly support a role for Cys 50 and Cys 59 in ligation of the 4Fe-4S cluster, and together with Cys 47 and Cys 53, they comprise an unusually compact motif with high identity to a motif ($CX_2CX_2CX_{4-7}C$) present in homologous Isf sequences. The conservation of this motif in Isf homologues

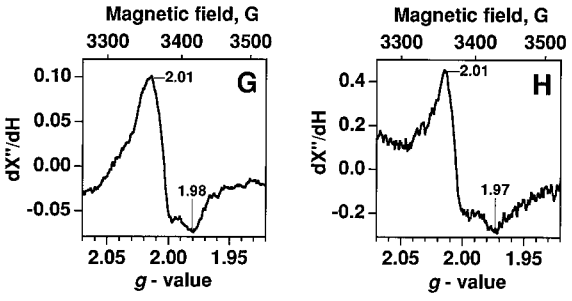

FIG. 6—*Continued.*

from metabolically diverse species in the domains *Bacteria* and *Archaea*, representing the extremes of evolution, is consistent with all four cysteines of the motif serving a common function. Nonetheless, EPR spectra for the reconstituted C47A and C53A variants indicated the presence of $[4Fe\text{-}4S]^{1+}$ clusters which could be used as an argument against involvement of these residues in ligation of the iron-sulfur center. The linewidths and apparent *g* values for the spectra of C47A and C53A (Fig. 6D and E; Table 1) differ significantly from those of wild-type Isf (Fig. 6A and B). This difference implies that either the ligands to the 4Fe-4S clusters in C47A and C53A changed or there was an overall change in the protein conformation. The occurrence of 4Fe-4S clusters in the C47A and C53A variants could be explained by other residues replacing Cys 47 and Cys 53 in a process called ligand swapping, for which there is precedent in other iron-sulfur proteins (14). Although ligand swapping is one possibility, it is also possible that 2-mercaptoethanol is an external thiolate ligand replacing Cys 47 and Cys 53 in these variants for the following reasons. First, reconstitution of the variants required 2-mercaptoethanol, a compound which has been shown to serve as an external ligand to the 4Fe-4S cluster in the C51D and C14G variants of the PsaC subunit of photosystem I in *Synechococcus* sp. strain PCC 7002 (2, 11). Second, the reconstitution conditions used in this work were nearly identical to the conditions used to reconstitute iron-sulfur clusters in PsaC. Clearly, a more rigorous investigation, most likely involving other techniques, is needed to determine the role of Cys 47 and Cys 53 in ligation of the 4Fe-4S cluster and the possibility of ligand swapping occurring in the C47A and C53A variants.

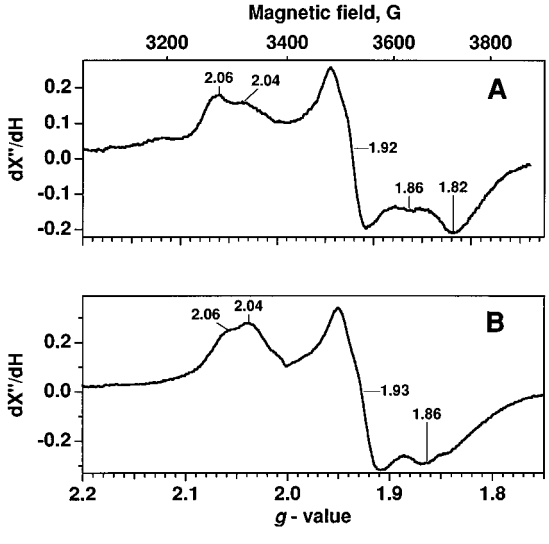

FIG. 7. EPR spectra of reduced reconstituted C16S (A) and C180S (B) variants. EPR conditions: temperature, 15 K; microwave power, 20 mW; modulation amplitude, 10 G.

The compact nature of the cysteine motif proposed to coordinate the 4Fe-4S cluster in Isf is unusual compared to motifs known to coordinate low-potential redox-active 4Fe-4S clusters where one of the cysteines are located remote in the sequence from the other three (typically CX_2CX_2C and a distant C). Thus, it is possible that each of the two 4Fe-4S centers in Isf is coordinated by three cysteines in the motif from one subunit and the fourth cysteine from the adjacent subunit. Examples of 4Fe-4S clusters bridging protein subunits are the nitrogenase Fe-protein and the F_X cluster in photosystem I (8, 9). If the iron-sulfur clusters bridge the subunits in Isf, this would be the first example of a protein with two bridging iron-sulfur clusters. Unfortunately, the failure of approaches to separate the subunits with iron-sulfur clusters intact precluded resolution of this question.

In addition to the proposed motif ligating the 4Fe-4S cluster, two other cysteines (Cys 16 and Cys 180) are present in the Isf sequence (Fig. 1). Similar properties (including linewidths and *g* values of the EPR signals) between the C16A/S variants and wild type serve as clear evidence that Cys 16 is not involved in coordination of the 4Fe-4S cluster. It should be noted, however, that variant C16A was produced in inclusion bodies, suggesting that this residue is important for proper folding of Isf. The C16S variant was produced as a soluble protein, and sequence comparisons (Fig. 1) indicate that a threonine residue in Isf homologues replaces Cys 16 of the *M. thermophila* Isf. These results suggest that a hydroxyl or sulfhydryl group is important in this position for maintaining a native conformation. Although the evidence supports noninvolvement of Cys 16 in ligation of the 4Fe-4S cluster, the evidence for noninvolvement of Cys 180 is weaker. The presence of C180A/S in inclusion bodies and instability of the reconstituted variants indicates improper folding which could be due to the loss of Cys 180 as a ligand to the 4Fe-4S cluster or an overall conformational change in the protein due to substitution of Cys 180. There is a possibility that Cys 180 is a ligand for about 50% of the clusters, accounting for the two distinct EPR species in the wild type. This possibility, however, is weakened by the fact that, in addition to the major species, there is a minor species in the spectra of the C180A/S variants with at least one of the three apparent *g* values ($g = 2.06$) that is clearly equivalent to one of the two species present in the wild type. Another possible explanation would be that substitution of Cys 180 changes the overall protein conformation, making one of the two protein conformations present in the wild type more preferable than the other. Hence, we observe one of the species present in the wild-type Isf, with only a minor contribution from the other. Unfortunately, our results do not allow us to unequivocally distinguish between these two possibilities.

This investigation has also provided insight into 4Fe-4S cluster and FMN self-assembly for Isf in vitro. The results show that insoluble wild-type apo-Isf can be solubilized and refolded to a conformational state that can be reconstituted with the 4Fe-4S cluster and FMN which is reduced with ferredoxin A at a rate comparable to as-isolated Isf. The results further show that both a 4Fe-4S cluster and FMN must be incorporated to adopt the native conformation. The presence of arginine was essential to achieve the conformational state necessary for reconstitution. It is proposed that arginine helps to reshuffle molecules trapped in nonproductive reactions, which results in increased refolding efficiency (5). The EPR properties of reconstituted wild-type Isf indicated that the procedure for re-

folding and reconstituting apo-Isf yielded a 4Fe-4S cluster with an environment identical to that for the wild type, suggesting that reconstituted Isf has a conformation similar, if not identical, to that of as-purified Isf. The ability to reconstitute apo-Isf in vitro provides a tool for investigating properties of the iron-sulfur cluster and FMN, for example, reconstitution with flavin analogs to probe the function of FMN. The ability to refold and reconstitute apo-Isf suggests that accessory factors are not essential for in vivo synthesis of Isf; however, the results showed that in vitro reconstitution was mostly incomplete, suggesting that a significant fraction of apo-Isf was improperly refolded. This result, and the requirement for arginine, suggests the possibility that a chaperonin may be important for efficient folding in vivo. It also cannot be ruled out that accessory proteins are necessary for efficient incorporation of the 4Fe-4S cluster and FMN.

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