Dodecins are so far the smallest known flavoproteins (68–71 amino acids) and are most likely involved in prokaryotic flavin storage. The dodecin monomers adopt a simple ββββ-fold and assemble to hollow sphere-like dodecameric complexes. Flavin binding by the dodecin from Thermus thermophilus showed a 1:1 stoichiometry and apparent dissociation constants in the range of biological redox reactions, such as the dehydrogenation of NAD(P)H, lipid esters, and D-amino acids, the oxidation of amines to imines, the formation and cleavage of disulfide bonds, the hydroxylation of aromatic substrates, or the activation of amines to imines, the formation and cleavage of disulfide bonds. The dodecin monomers adopt a simple ββββ-fold and assemble to hollow sphere-like dodecameric complexes. Flavin binding by the dodecin from T. thermophilus showed a 1:1 stoichiometry and apparent dissociation constants in the submicromolar to nanomolar range as characterized by isothermal titration calorimetry and fluorescence titrations. The structures of the flavin-prebound and FMN-reconstituted state of the T. thermophilus dodecin revealed binding of FMN dimers in a novel si-si- rather than the re-re-orientation of their isoalloxazine moieties as found before in an archaeal dodecin. Electron paramagnetic resonance studies demonstrated that upon reduction the excess electron is localized only on one flavin, thus making dodecin-bound flavins highly refractory to redox chemistry. Besides FMN dimers, trimers of coenzyme A are additionally bound to this eubacterial dodecin along the 3-fold symmetry face II of the dodecin complex. Therefore, dodecins can act as bifunctional cofactor storage proteins that sequester catalytic cofactors in prokaryotes very efficiently in an aggregated and unreactive state.

Flavoproteins are ubiquitous proteins using flavins as prosthetic groups. Because riboflavin (vitamin B₂) serves mostly as a biosynthetic intermediate, FMN and FAD are the principal flavin cofactors (1). They are involved in the catalysis of a wide range of biological redox reactions, such as the dehydrogenation of NAD(P)H, lipid esters, and D-amino acids, the oxidation of amines to imines, the formation and cleavage of disulfide bonds, the hydroxylation of aromatic substrates, or the activation of molecular oxygen. Furthermore, flavoproteins serve as electron transmitters in electron transfer processes like oxidative phosphorylation. Here, they act as mediators between typical 2-electron donors like NADH and 1-electron acceptors like the heme group. This versatility to engage in one- and two-electron transfer reactions is due to their isoalloxazine moiety that adopts either an oxidized, a semiquinoid, or fully reduced redox state (2). Flavins are not only involved in redox reactions but also in the sensing of blue or ultraviolet light (3). In cryptochromes, flavin chromophores mediate the flowering and daily light/dark cycles in plants (4), in phototropins they regulate phototropism (5), and in photolyases they are involved in DNA repair (6). Beside catalytic or sensoric actions, flavoproteins are also involved in the binding or transport of flavins. During pregnancy, a specific carrier system evolves in vertebrates including the riboflavin-binding proteins (RCPs or RIBPs), since adequate amounts of vitamin B₂ are essential for the normal fetal development (7).

Dodecins are a novel family of flavin-binding proteins of unknown function that were discovered in the Archaea Halobacterium salinarum during an inverse structural genomics project on halophilic proteins (8). So far it is the smallest known flavoprotein with only 68 amino acids. Apart from halarchaea, dodecins are found in many eubacterial genomes, as 16% of all completely sequenced eubacteria possess dodecin encoding genes (84 of 520 total, as of July 2007). These organisms constitute mainly proteobacteria (64 of 273) but also actinobacteria (14 of 47), chlorobi (4 of 16), and deinococcus/thermus species (2 of 4), which thrive either in soil or aquatic habitats or as parasites in host organisms. Interestingly, dodecins occur in a large variety of pathogenic bacteria (e.g., Pseudomonas aeruginosa, Mycobacterium tuberculosis) as well as in organisms known for their biotechnological and environmental relevance (e.g., Chlorobium tepidum, Geobacter sulfurreducens). Based on sequence conservation, dodecins can be divided into two groups; whereas eubacterial dodecin sequences share an average sequence identity of 53%, the archaeal dodecin sequences cluster separately with only 31% sequence identity to the eubacterial dodecins. The crystal structure of the halophilic dodecin complexed to riboflavin shows a dodecameric hollow sphere-like arrangement of monomeric subunits with cubic 23 symmetry. Flavins are bound as dimers along the interfacial surfaces of the dodecameric complex. The main stabilizing interactions between the flavin dimer and the dodecin complex occur via an aromatic tetrad involving the isoalloxazine groups of the flavin dimer and the indole groups of two neighboring tryptophan residues. The dodecin monomer exhibits a new protein fold adopting a simple βαββ topology, where the amphipathic α-helix is partly enwrapped by the three-stranded antiparallel β-sheet. Structural studies and fluorescence-based binding experiments for the H. salinarum dodecin (9) showed the binding of several...
kinds of flavins and flavin-like molecules. Furthermore, lumiflavin and lumichrome bind like FMN and riboflavin as stacked dimers in the flavin binding pockets with 1:1 stoichiometries, whereas FAD is bound in a U-shaped locked conformation with an apododecin/ligand ratio of 2:1 (10). Binding constants obtained by fluorescence titrations indicate preferred binding of small flavins and flavin-like molecules such as lumiflavin and lumichrome compared with the bulkier flavins FMN and FAD.

We have characterized the dodecin from *Thermus thermophilus*, previously assigned as hypothetical protein TTHA1431, in terms of its structure and flavin binding characteristics. Besides a surprisingly different mode of flavin dimer binding compared with the *H. salinarum* dodecin, the binding of an additional cofactor, coenzyme A, was observed in *T. thermophilus* dodecin suggesting that dodecins have evolved to form highly efficient storage proteins for sequestering multiple cofactors in prokaryotic organisms.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification**

Using *T. thermophilus* HB8 genomic DNA and a set of primers (5'-GCGGCCCATGGGCAAGGTCTACAAGAAGG-3' and 5'-CGGCTCAGCTAAGTCTCTTCAAGCGGAACT-3') the dodecin gene (GenBank™ accession number 3168700) was amplified by PCR and cloned into the PET-28a(+) vector (Invitrogen) via the NcoI/XhoI sites. The R45A and R65A mutants were created using the QuikChange site-directed mutagenesis kit (Stratagene) and the mutagenesis primers 5'-CGGCTCGGCAAGGTCTACAAGAAGG-3' for the R45A mutant and the primers 5'-CTAAGTCTCCTTCAAGCGGAAC-3' and 5'-CTGGAGGTGGGGTTCGCT-3' for the R65A mutant. After verification by DNA dyeo-seqencing, mutant proteins were expressed and purified as described for the wild type protein.

For recombinant *T. thermophilus* dodecin production, *Escherichia coli* strain BL21(DE3)Gold was transformed with the appropriate plasmid and selected with 35 μg/ml kanamycin. 2 liters of LB medium were inoculated with 50 ml of an overnight culture and grown at 150 rpm and 37 °C for 20 h until an A₅₉₅ of 4.5 was reached to promote autoinduction (11). The cells were pelleted, resuspended in 20 mM Tris/HCl, pH 8.0, 200 mM NaCl, and stored at −80 °C.

The frozen cells were thawed and disrupted by a French press. The cell lysate was centrifuged (15,000 rpm, 30 min), and the supernatant was incubated at 65 °C for 10 min. Heat-denatured proteins were removed by centrifugation (15,000 rpm, 30 min), and the supernatant was dialyzed against 20 mM Tris/HCl, pH 8.0, overnight. The protein was further purified by anion-exchange chromatography using a Q-Sepharose high performance (Amersham Biosciences) column equilibrated with 20 mM Tris/HCl, pH 8.0, and a linear gradient of 0–1 M NaCl in 20 mM Tris/HCl, pH 8.0. Fractions containing the *T. thermophilus* dodecin were pooled, concentrated, and applied to a Superdex 200 (Amersham Biosciences) gel filtration column (120 ml) that was equilibrated with 20 mM Tris/HCl, pH 8.0, 100 mM NaCl. Fractions of the dodecameric dodecin with more than 90% purity were concentrated to about 10 mg/ml and stored at −20 °C. Protein concentrations were determined by the Bradford protein assay (Bio-Rad).

Apododecin was prepared by an unfolding/refolding protocol. The *T. thermophilus* dodecin obtained from anion exchange chromatography was denatured at a concentration of 1 mg/ml with 6 M guanidinium chloride and dialyzed 3 times against 6 M guanidinium chloride, 20 mM Tris/HCl, pH 8.0, and refolded by dialyzing two times against 20 mM Tris/HCl, pH 8.0. Alternatively, the protein was precipitated with 6% trichloroacetic acid followed by dissolving the pellet in 6 M guanidinium chloride, 20 mM Tris/HCl, pH 8.0, and dialyzed stepwise against 20 mM Tris/HCl, pH 8.0, 100 mM NaCl. In both cases aggregated protein was removed by size exclusion chromatography.

**Protein Characterization**

Electrospray ionization mass spectra were taken with an Agilent 1100 Series mass spectrometer to obtain the molecular mass of the dodecin monomer. The apparent molecular mass of the oligomeric assembly was determined by gel filtration chromatography on a Superdex 200 HR10/30 (Amersham Biosciences) column equilibrated with 20 mM Tris/HCl, pH 8.0, 100 mM NaCl. Here, 200 μl of *T. thermophilus* dodecin with a concentration of 1.1 mg/ml were loaded onto the column and eluted with a flow rate of 0.5 ml/min. To obtain a calibration line, mixtures of various size standards (Amersham Biosciences) were loaded.

**Cofactor Identification**

Bound flavin cofactors were identified by HPLC/electrospray ionization-time-of-flight. After releasing the cofactor by denaturing a dodecin solution with 6% trichloroacetic acid, the cofactor extracts as well as FMN and riboflavin standards were loaded onto a Nucleodur 2 × 125-mm C18 gravity column with a particle size of 3 μm (Macherey & Nagel) and eluted with a water/methanol gradient at a flow rate of 0.2 ml/min. The elution was monitored by UV absorption at 215 nm, and masses were analyzed by electrospray ionization-time-of-flight using a Q-Star pulser (Applied Biosystems).

Cofactor concentrations were determined spectrophotometrically using the following molar extinction coefficients: 11,300 M⁻¹ cm⁻¹ at 450 nm for FAD, 12,600 M⁻¹ cm⁻¹ at 448 nm for FMN, 12,300 M⁻¹ cm⁻¹ at 448 nm for riboflavin, 10,900 M⁻¹ cm⁻¹ at 444 nm for lumiflavin (12), and 8,200 M⁻¹ cm⁻¹ at 354.5 nm for lumichrome (9).

**Cofactor Reconstitution**

To obtain cofactor-reconstituted dodecin, a saturated flavin solution was added to the protein solution in a concentrator cell (Vivascience) until the flow-through became yellow. Reconstituted *T. thermophilus* dodecin was separated from the excess of flavin by gel filtration chromatography. For an estimation of the cofactor concentration, the protein was precipitated with 6% trichloroacetic acid, and the absorption of the supernatant at

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2 The abbreviations used are: HPLC, high performance liquid chromatography; ITC, isothermal titration calorimetry; ENDOR, electron-nuclear double resonance; mT, milli-Tesla.
**The Dodecin from T. thermophilus**

**TABLE 1**

| Data collection and refinement statistics |
|------------------------------------------|
| r.m.s.d., root mean square deviation; a.u., per asymmetric unit. |
| Data set     | R65A/FMN                  | R45A/FMN                  | Wild type/FMN | Wild type          |
| X-ray source | BW7A/DESY                 | FR591/CuK<sub>a</sub>    | FR591/CuK<sub>a</sub> | FR591/CuK<sub>a</sub> |
| Wavelength (Å) | 0.9774                   | 1.54179                  | 1.54179       | 1.54179            |
| Space group   | P3,21                     | P2<sub>1</sub>, 2, 2      | P2<sub>1</sub>, 2, 2 | P4<sub>1</sub>, 2, 2 |
| Unit cell (a, b, c in Å) | 67.59, 67.59, 67.59     | 67.29, 68.49, 229.89  | 67.48, 98.04, 157.60 | 65.76, 65.76, 202.41 |
| Resolution (Å) | 15.0–1.40                | 25–2.58                  | 24–2.60        | 19.9–2.40         |
| No. of structure factors | 373,150              | 122,382                  | 60,816         | 129,703            |
| Unique structure factors | 87,239               | 32,742                   | 22,085         | 18,248             |
| Mosaicity (°) | 0.37                     | 0.42                     | 1.00           | 0.49               |
| Completeness<sup>c</sup> | 19.7 (4.2, 0.079 (0.300) | 13.2 (3.59, 0.094 (0.242) | 19.0 (3.9, 0.080 (0.242) | 23.3 (3.9, 0.087 (0.362) |
| Solvent content | 0.502                    | 0.580                    | 0.506          | 0.487              |
| Dodecamers/a.u. | 0.5                      | 1                       | 1              | 0.5               |

| Refinement |
|------------|
| R-factor/R<sub>merge</sub> | 0.170; 0.193 (0.214; 0.228) | 0.206; 0.234 (0.361; 0.336) | 0.219; 0.244 (0.373; 38.8) | 0.225; 0.262 (0.277; 0.278) |
| r.m.s.d. bond lengths (Å), angles (°) | 0.008; 1.427 | 0.007; 1.008 | 0.009; 1.277 | 0.008; 0.960 |
| No. of water molecules | 509 | 36 | 25 | 13 |
| Average B-factor (Å<sup>2</sup>) | 12.9 | 30.3 | 52.0 | 64.3 |

<sup>a</sup> Values in parentheses correspond to the highest resolution shell.

<sup>b</sup> R<sub>merge</sub> = Σ||F<sub>obs</sub>|| – Σ||F<sub>calc</sub>||/Σ||F<sub>obs</sub>||.

<sup>c</sup> R<sub>merge</sub> = Σ||F<sub>calc</sub>||/Σ||F<sub>calc</sub>||.

<sup>d</sup> R<sub>free</sub> = crystallographic R-factor based on 1.6% (R65A), 3.1% (R45A), 5.1% (wild type/reconstituted), and 5.9% (wild type) of the data withheld from the refinement for cross-validation.

A<sub>448</sub> was measured. Using the extinction coefficient of free FMN and a modified extinction coefficient of FMN in 6% trichloroacetic acid (ε<sub>448</sub> = 10,400 M<sup>−1</sup> cm<sup>−1</sup>) the extinction coefficient of dodecin-bound FMN was determined to be 9,500 M<sup>−1</sup> cm<sup>−1</sup>. A calculated FMN:protein ratio of 1:1.2 was consistently obtained for different preparations indicating that FMN was bound in a 1:1 stoichiometry.

**Crystallization and Data Collection**

Light orange-colored crystals of non-reconstituted *T. thermophilus* dodecin were grown using hanging drop vapor diffusion techniques at 20 °C from 7 mg/ml protein solution in gel filtration buffer. Crystals were obtained by equilibration of a mixture containing 1 µl of protein solution and 1 µl of reservoir solution (0.2 M magnesium acetate, 0.1 M sodium cacodylate, pH 6.5, 30% 2-methyl-2,4-pentanediol) against 750 µl of reservoir solution. Tetragonally shaped crystals (space group P4<sub>1</sub>2<sub>1</sub>2<sub>1</sub>, a = b = 65.76 Å, c = 202.14 Å, one hexamer per asymmetric unit) appeared within several weeks. FMN-reconstituted dodecin (5 mg/ml with 5% glycerol) was crystallized in 0.2 M sodium citrate, 0.1 M Tris/HCl, pH 7.5, 25% polyethylene glycol 400. Dark orange crystals (P2<sub>1</sub>, 2<sub>1</sub>, 2<sub>1</sub>, a = 67.48 Å, b = 98.04 Å, c = 137.60 Å, one dodecamer per asymmetric unit) appeared within a few weeks of incubation. The FMN-reconstituted R45A mutant (7.5 mg/ml) crystallized in 0.1 M ammonium sulfate, 0.05 M sodium cacodylate, pH 6.5, and 15% polyethylene glycol 8000. Here the space group was P2<sub>1</sub>, 2<sub>1</sub>, 2<sub>1</sub> (a = 67.29 Å, b = 68.49 Å, c = 229.89 Å), with one dodecamer per asymmetric unit.

Orange-colored, hexagonally shaped crystals of the FMN-reconstituted R65A mutant (P3<sub>2</sub>, 2<sub>1</sub>, a = b = 67.59 Å, c = 169.19 Å, half a dodecamer per asymmetric unit) appeared under sitting-drop conditions within a few weeks. The sitting drop consisted of 300 nL of 26 mg/ml dodecin and 300 nL of reservoir solution (0.4 M ammonium phosphate), and the reservoir volume was 100 µL. Data sets of the wild type dodecins and the R45A mutant were collected at 100 K from flash-frozen crystals using a Bruker-Nonius FR591 (50 kV, 80 mA)-rotating CuK<sub>a</sub> anode with a MAR imaging plate (MAR-Research). For the R65A mutant, a 1.4-Å dataset was collected at beamline BW7A at EMBL outstation, Hamburg. For the non-reconstituted dodecin, a 2.4-Å data set was recorded, and the crystals of the reconstituted dodecin diffracted up to 2.6 Å (Table 1). The data sets were processed using MOSFLM and scaled with SCALA (13). All structures were solved by molecular replacement with MOLREP (14) using the *H. salinarum* dodecin structure as an initial search model and refined by REFMAC5 (15) and COOT (16) using restrained TLS refinement.

**Spectroscopic Measurements**

Absorption spectra from 200 to 700 nm were recorded using an Ultrospec 3100 spectrophotometer (Amersham Biosciences) at room temperature with a scan rate of 350 nm/min and 0.5-nm steps. Buffer spectra were recorded under identical conditions and subtracted afterwards.

Fluorescence emission spectra were recorded with a FP-6500 spectrofluorometer (Jobin Yvon) equipped with a Jasco ETC-273T Peltier-type temperature control system at 20 °C using a 200-µl quartz cuvette with an excitation wavelength of 450 nm and an emission wavelength of 460–700 nm. Spectra were taken with a scan rate of 1000 nm/min in 0.5-nm steps. For fluorometric measurements, filtered protein and cofactor solutions with a concentration of 5 µM (related to flavin concentration) in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl were used.

Circular dichroism measurements were carried out on a J-810 (Jobin Yvon) equipped with a PTC-423S Peltier-type temperature control system using quartz cuvettes with 0.1-cm path lengths. Protein samples had concentrations of 12.5 mM in 10 mM Tris/HCl, pH 7.5. Data were recorded from 190 to 280 nm with 0.2-nm steps at a scan rate of 100 nm/min and averaged from 3 to 5 scans recorded at 20 °C. The baseline was corrected by subtracting the spectrum of the buffer collected under the same conditions. Thermal unfolding curves were measured by...
following the ellipticity change at 198 nm during continuous heating from 20 to 95 °C at a scan rate of 2 °C/min.

**Determination of FMN Binding**

Flavins (Sigma) with a purity of >95% were used without further purification. Lumichrome (Sigma) required further purification by HPLC. Samples were separated on an Agilent 1100 HPLC system using a Nucleodur 125/2 C18ec column with a 3-μm particle size at a flow rate of 0.2 ml/min and a column temperature of 40 °C with a linear gradient of 10–80% methanol, 0.1% trifluoroacetic acid in water, 0.1% trifluoroacetic acid within 25 min. Pooled fractions of interest were lyophilized.

Dissociation constants of flavin-dodecin complexes were determined by fluorescence titrations, where the fluorescence quench of the flavin ligand upon binding to the *T. thermophilus* dodecin was monitored with a FP-6500 spectrofluorometer (Jasco) equipped with a PTC-423S Peltier-type temperature control system. In this experiment 11.5 μM dodecin containing endogenously bound FMN was used to avoid the kinetic effect observed before by ITC measurements and fluorescence titrations where apododecin was used. Ligand concentrations were 200 nM (FAD, lumichrome), 203 nM (riboflavin), 198 nM (FMN), and 232 nM (lumiflavin). All solutions were sterile-filtered and pre-equilibrated at 20 °C in the dark. Measurements were made in 20 mM Tris/Cl, pH 8.0, 100 mM NaCl.

Fluorescence titrations were performed at 20 °C in a 2-ml round cuvette. In a typical titration experiment, 1000 μl of flavin solution was titrated with 4-μl aliquots of dodecin every 2 min under stirring until an end point was reached. For riboflavin, FMN and lumiflavin, emission at 529 nm after excitation at 450 nm was measured with medium sensitivity and slit widths of 10 nm for emission and excitation. In the titration using FAD, the parameters were set to 450/532 with slit widths of 5 nm (excitation) and 10 nm (emission) and high sensitivity due to low FAD fluorescence at pH 8.0. In the case of lumichrome, the emission at 460 nm after excitation at 380 nm with slit widths of 5 nm (excitation) and 10 nm (emission) were measured. For the correction of the fluorescence of endogenously bound FMN, the protein was titrated into buffer under the same conditions as in the binding experiments.

The fluorescence was normalized to the fluorescence start point and corrected for the fluorescence of the first dimer. Overall yields were 17 mg of *T. thermophilus* dodecin per liter of culture with a purity greater than 95% (Fig. 1B). Mass spectrometric analysis of the recombinant *T. thermophilus* dodecin revealed a molecular mass of 7618.1 Da compared with 7616.6 Da as calculated for residues Gly-2 to Thr-69. Accordingly, the N-terminal formyl-methionine was removed by post-translational modification.
The Dodecin from *T. thermophilus*

![Graph](image)

**FIGURE 1. Flavin binding by the oligomeric *T. thermophilus* dodecin.** A, size exclusion chromatography of 200 μl of 1.1 mg/ml dodecin with endogenously bound FMN (dashed lines) and after reconstitution with an excess of flavin (dotted lines). Peaks 1 and 2 correspond to oligomeric dodecin species, and peak 3 corresponds to an excess of flavin. B, SDS-PAGE analysis of the purified *T. thermophilus* dodecin after gel filtration. Lane 1, non-reconstituted dodecin; Lane 2, reconstituted dodecin; Lane 3, molecular weight markers. The molecular weight marker was pasted from another position of the same gel. C, absorption spectra of dodecin-bound FMN (solid line) and free FMN (dashed line). Spectra are plotted relative to each other with respect to the molar absorptivity coefficients for FMN (ε₄₅₀ = 12600 M⁻¹ cm⁻¹) and dodecin bound FMN (ε₄₅₀ = 9500 M⁻¹ cm⁻¹).

Spectroscopic Characterization of the Dodecin-Flavin Complex—UV/VIS spectroscopy of the orange-colored *T. thermophilus* dodecin complex showed the characteristic absorption spectrum of oxidized flavin species with absorption maxima at 270, 380, and 450 nm (Fig. 1C). An additional slightly red-shifted shoulder was observed in the range of 475–480 nm, indicating vibrational fine structure resulting from tight cofactor binding in the protein environment. Moreover, there is significant absorbance up to 600 nm representing charge-transfer interactions of the isodolexazine rings with neighboring aromatic residues. According to fluorescence spectroscopy, the flavin fluorescence was quenched by >98% when compared with flavins in free solution (see Fig. 3A). Because the residual fluorescence was indistinguishable from free flavin, it is obvious that the binding of flavins to the dodecin oligomer almost completely quenches the endogenous flavin fluorescence. Likewise, attempts to induce a triplet state of the dodecin-bound flavin species in the frozen state failed when the dodecin complex was excited by a continuous 450-nm pump laser with triplet formation being followed by EPR (data not shown). Ultrafast UV-visible spectroscopy on defined flavin-dodecin complexes finally showed that a rapid electron transfer from neighboring tryptophan residues to the isodolexazines takes place in the excited singlet state, thus avoiding consequent intersystem crossing and causing the efficient quench of flavin fluorescence.³

Cofactor Binding and Oligomerization of the Dodecin Complex—The electrospray ionization-mass spectroscopy analysis of the flavin cofactor extracted under denaturing conditions from the dodecin complex showed by comparison with FMN, riboflavin, and FAD standards that the recombinant *T. thermophilus* dodecin predominantly binds the negatively charged FMN. This finding contrasts with the characterization of the previously discovered dodecin from *H. salinarum*, which was found to copurify exclusively with stoichiometrically bound riboflavin when being isolated from the cytosol of this haloarchaeon (8). The purified *T. thermophilus* dodecin complex exhibited a protein:cofactor ratio of 1:0.2, indicating substoichiometric flavin binding to the recombinant *T. thermophilus* dodecin, most likely due to the lack of sufficient flavin supply in the *E. coli* cytosol. However, the dodecin complex could be reconstituted afterward in vitro with either FMN, FAD, or riboflavin as shown by apparent cofactor:protein ratios of 1:1.2 after size exclusion chromatography, suggesting the formation of stoichiometric dodecin-flavin complexes.

To determine the oligomeric state of the dodecin complex, analytical size exclusion chromatography was performed as shown in Fig. 1A. Peak 1 indicated a molecular mass of 96.4 kDa, whereas peak 2 corresponded to a molecular mass of 39.8 kDa, suggesting that dodecameric *T. thermophilus* dodecin is in equilibrium with a hexameric state, if only substoichiometric amounts of FMN are bound. Reconstitution with FMN or riboflavin caused the disappearance of peak 2 indicating a stabilization of the dodecameric complex. SDS-PAGE analysis of the purified dodecin showed a high resistance against denaturation by SDS or heat. Even after boiling the protein samples for 5 min in gel-loading buffer comprising 4% (w/v) SDS, the monomer band was accompanied in the SDS-PAGE by an oligomeric band at the apparent molecular weight of a hexameric protein species (Fig. 1B). Whether this band corresponds to a partly denatured hexameric or a non-denatured dodecameric complex is unclear. As expected for a thermophilic protein the *T. thermophilus* dodecin oligomer exerts high thermal stability, since CD spectra of the holocomplex at 20 and 95 °C as well as melting curves measured from 20 to 95 °C showed no significant loss of secondary structure. Interestingly, flavin removal did not affect the thermal stability as no melting of the dodecameric apododecin was observed by CD spectroscopy.

Redox Chemistry of the *T. thermophilus* Dodecin—To characterize the redox activity of dodecin, which might be governed by the unique stacking arrangement of the flavin chromophores, chemical reduction of the *T. thermophilus* dodecin was attempted using reductants such as EDTA, dithiothreitol, and sodium dithionite. Although these reagents did not yield detectable reduced flavin species, reduction with titanium(III) citrate finally produced minor amounts of the flavin semiquinone species that remained stable at liquid nitrogen conditions. By continuous-wave EPR, a radical signature centered at g = 2.0035(5) was detected that is characteristic for a flavin radical (18–20) (Fig. 2A). After the discussion outlined previously (18), neutral flavin radicals (FADH⁻) show typically peak-to-peak EPR line widths of about 2.0 mT, whereas anionic radicals show a reduced line width down to about 1.3 mT as a result of deprotonation of N(5) (21, 22). Experimentally, a peak-to-peak line width of 1.3 mT was observed, which is consistent with the latter. However, line-width reductions are also expected for

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³ G. G. Gurzadyan, B. Meissner, L. O. Essen, and M. E. Michel-Beyerle, manuscript in preparation.
The Dodecin from *T. thermophilus*

phenomena due to delocalization or very fast electron transfer were first observed in EPR studies of the "special pair" of bacteriochlorophylls in bacterial photosynthetic reaction centers (23) and, more recently, in the circular arrays of bacteriochlorophylls of the light-harvesting complexes LH1 and LH2 (24). To distinguish between the various mechanisms for the EPR line narrowing of the dodecin-bound flavin radical species, pulsed proton ENDOR experiments were performed (Fig. 2B). Despite moderate spectral resolution due to the low concentration of the flavin radical in *T. thermophilus* dodecin, a pair of lines of axial symmetry was clearly observed and assigned to the hyperfine coupling of the methyl protons attached to C(8α); \( A_{\perp} = 9.8 \text{ MHz} \), \( A_{\parallel} = 11.7 \text{ MHz} \), yielding \( A_{\text{iso}} = 10.4 \text{ MHz} \). When compared with other flavoprotein radicals, either anionic or neutral, this value is rather large, thus excluding the dimeric nature of the flavin species for which all hyperfine couplings should be halved if delocalization between the flavin units is uniform (23). Accordingly, electronic coupling of the coplanar pair of flavins in the radical form appears to be rather small. Hence, the observed narrow line width in the continuous-wave-EPR spectra is due to the anionic nature of the flavin. This assignment is also corroborated by the experimental hyperfine coupling that is in a range typically expected for 8α-methyl protons in flavin anion radicals (25). Taking together the findings that dodecin is only difficult to reduce both chemically and photochemically and that the semireduced state is not stabilized by delocalization, it is rather unlikely that dodecins are involved in biological redox processes.

**Specificity and Time Dependence of Flavin Binding to the *T. thermophilus* Dodecin**—To determine binding affinities between the apo form of *T. thermophilus* dodecin and the flavin cofactor FMN, fluorescence titrations as well as ITC measurements were carried out using apododecin prepared by a denaturation/refolding protocol. For fluorescence titration, the emission at 530 nm after excitation at 450 nm was detected, exploiting the fluorescence quench occurring upon flavin binding (Fig. 3A).

dimers or N-mer of electronically interacting flavins, when the unpaired electron spin is either delocalized over the then-extended conjugated \( \pi \)-electron system or when the spin is rapidly transferred between the nitrogen flavins. This limiting value of the line width in an N-mer is by a factor of \( N^{1/2} \) smaller than the monomer line width, where \( N \) is the number of sites over which charge migration occurs. Such line narrowing...
To investigate the binding of the remaining dimers, the *T. thermophilus* dodecin in its flavin-pre-bound state was used for fluorescence titrations, which has endogenously bound 20% FMN after purification. Here, binding of additional flavin to the *T. thermophilus* dodecin showed no measurable time dependence. For the binding affinity between FMN and *T. thermophilus* dodecin, an apparent $K_D$ of 0.311 µM was determined with a 1:1 stoichiometry (Fig. 3, B and C). Besides FMN, also FAD, riboflavin, lumiflavin, and lumichrome were tested for their binding affinities to the *T. thermophilus* dodecin. All flavins showed similar binding affinities, which are 589 ± 26 nm for FAD, 311 ± 18 nm for FMN, 233 ± 37 nm for riboflavin, 141 ± 11 nm for lumiflavin, and 80 ± 3 nm for the lumichrome-dodecin complex (Fig. 3C). This is in contrast to the binding affinities reported for the *H. salinarum* dodecin (9). Here, the small ligands lumichrome, lumiflavin, and riboflavin showed significantly lower dissociation constants (11.6, 17.6, 35.8 nm) than the bulkier flavins FMN and FAD (11.3 and 0.44 µM). Furthermore, FAD was found to bind in a 1:2 stoichiometry to the *H. salinarum* dodecin (9), which could be attributed to the binding of a closed conformer of FAD within the flavin-binding site of *H. salinarum* dodecin (10), whereas in the *T. thermophilus* dodecin FAD is bound like the other flavins with 1:1 stoichiometry.

**Overall Structure of the *T. thermophilus* Dodecin—**The *T. thermophilus* dodecin and its R45A and R65A mutants crystallized in four different crystal forms. Tetragonal crystals (space group P4₁2₁2) were obtained for the complex between wild type *T. thermophilus* dodecin and substoichiometrically bound amounts of FMN. Orthorhombic crystal forms (space group P2₁2₁2₁) were generated for the stoichiometric 1:1 complexes with FMN, which differed between the wild type and the R45A dodecin in the dimensions of the $b$ and $c$ axes, whereas the R65A mutant crystallized in the trigonal space group P3₁2₁2₁.

The *T. thermophilus* dodecin structures were solved by molecular replacement starting from the structure of the *H. salinarum* dodecin (34% sequence identity) as a search model (8). All crystal forms of the *T. thermophilus* dodecin showed the characteristic dodecameric assembly of monomeric subunits (Fig. 5, C and D), as anticipated before from analytical size exclusion chromatography. The *T. thermophilus* dodecin dodecamer with its cubic 23 point symmetry adopts a hollow sphere-like shape with an inner diameter of 23 Å and an analytical size exclusion chromatography. The *T. thermophilus* dodecin dodecamer with its cubic 23 point symmetry adopts a hollow sphere-like shape with an inner diameter of 23 Å and an
outer diameter of 60 Å. The monomeric subunits have overall dimensions of $44 \times 20 \times 17$ Å and adopt a simple $\beta_1\alpha_1\beta_2\beta_3$ topology, that is characteristic of the dodecin-fold (SCOP classification), where the $\alpha$-helix is partly enwrapped by the three-stranded antiparallel $\beta$-sheets (Fig. 5A). Although the $\alpha$-helices face the outer surface of the dodecameric complex, the $\beta$ sheets line the interior of the dodecamer. Structural comparison between the *T. thermophilus* and *H. salinarum* dodecins shows the high conservation of the dodecin fold with a root mean square deviation of 0.67 Å for 58 C$_\alpha$ atoms. Major structural differences are restricted to the variable $\beta_2$-$\beta_3$ loop and the N and C termini (Fig. 5B), which reside on the outer surface of the dodecin complex and are not involved in flavin binding. Likewise, the quaternary structure is similarly conserved as a superposition of the chains A of both dodecin complexes on each other leads to largest divergences in the superposition of the other chains of below 1.5 Å.

**The Flavin Dimer Binding Site of the *T. thermophilus* Dodecin—** The various crystal structures of the *T. thermophilus* dodecin show the binding of flavin dimers along the 2-fold axes of the cubic-symmetric oligomer. The binding site is made up along the interface of four different dodecin subunits and corresponds to a $10 \times 15$-Å large cleft (Fig. 5D) connecting the inner space of the hollow sphere-like particle with the exterior bulk.
The Dodecin from T. thermophilus

FIGURE 6. The flavin dimer binding site of the T. thermophilus dodecin. A, stereo diagram of the view along the 2-fold symmetry axis on the FMN binding site. Selected residues are highlighted, and interactions are shown as black dotted lines. The 2Fo Fobs map (contouring level 1.5 σ) is shown in green. B, schematic representation of the aromatic tetrade of T. thermophilus (left) in comparison to the H. salinarum dodecin (right). Flavin dimers are shown in gray, and interacting residues are shown in orange for the T. thermophilus dodecin and in green for the H. salinarum dodecin. Hydrogen bonds are shown as gray dotted lines. For the lower representation, the upper representation was turned by 90° to the front. This figure was prepared by PYMOL (32).

The free access to the flavin binding sites is consistent with the reversible flavin binding and exchange as observed for the T. thermophilus dodecin in its flavin-prebound state. In the crystal structure of the T. thermophilus dodecin, where only endogenously bound flavin is present, the electron density observed at the end of the ribityl side chains clearly indicated the presence of the FMN phosphate group and, thus, corroborated the previous electrospray ionization-mass spectroscopy results concerning the cofactor content of the T. thermophilus dodecin (Fig. 6A). Due to substoichiometric FMN binding, only two of the six putative binding sites within the dodecin dodecamer show electron density for flavin molecules with refined occupancies of 70%. The other four binding pockets appear to be almost devoid of bound flavin (occupancies of <50%) as they only show residual density at the expected positions of the flavin dimers. However, the binding mode of the flavin dimer is not affected by the substoichiometric binding, because in the fully reconstituted dodecin-FMN complex the orientation of the FMN molecules is unchanged.

Important interactions stabilizing the flavin dimers within the T. thermophilus dodecins are shown in Fig. 6. Interestingly, only few highly conserved residues are involved in binding of the flavin dimer. As is typical for flavoproteins, the dimethylbenzene ring is primarily surrounded by hydrophobic side chains, whereas the nucleotide part is stabilized by hydrogen bonds to adjacent polar residues. Val-11 and Val-59 surround the hydrophobic side of the flavin forming an un polar binding pocket. Val-59 is moderately conserved and can be mostly replaced by Ser, Thr, or Gly. Likewise, Val-11 can be replaced in several other dodecin homologues by either Ile or Thr (Fig. 7). The side chains of Gln-57 and Arg-45 contribute to the flavin binding by the formation of three hydrogen bonds. Gln-57 is a highly conserved residue present in all dodecin homologues, forming a hydrogen bond to C2-O und N3-H. Such hydrogen bonds between the apoprotein and the N3-H of the flavin cofactor are ubiquitously observed in other flavoprotein structures (26). Arg-45, stabilizing the flavin by hydrogen bonds to C4-O and N5, is conserved among eubacterial dodecins. In the archaeal dodecins, Arg-45 is substituted by Gly-45. The most important interaction is made by the indole groups of two highly conserved tryptophan residues (Trp-38) from two symmetry-related subunits. These residues stabilize the isoalloxazine moiety by π-π stacking, forming a sandwich-like aromatic tetrade. The binding of the isoalloxazine ring by π-π stacking is also known from other flavin binding proteins; in flavodoxins and riboflavin-binding proteins the flavin is stabilized by forming an aromatic triad where the flavin is sandwiched between a tryptophan and a tyrosine residue (7, 27).

The FMN phosphate group forms multiple salt bridges and hydrogen bonds to residues Lys-3, Tyr-5, Arg-65, and Asp-37. Whereas Tyr-5 is present in most dodecin homologues (53 of 56 sequences), only moderate conservation is observed for the residues Lys-3 (only 13 sequences), Arg-65 (35/55), and Asp-37 (19/57) (Fig. 7). Additional interactions by the ribityl group of FMN are only formed between the 2′-OH group and the main-chain carbonyl of Asp-37 and the 3′-OH group and the side chain of Tyr-5.

Comparison with H. salinarum Dodecin—Although the overall structure and the binding of flavin dimers appears to be similar in the dodecins from T. thermophilus and H. salinarum, the two dodecins differ significantly in their mode of flavin binding (Fig. 6B). Whereas the 1.7-Å structure of the H. salinarum dodecin clearly shows the presence of riboflavin dimers,
FIGURE 7. Sequence alignment of dodecin homologues. BLAST search was performed with NCBI-BLAST using the *T. thermophilus* dodecin sequence as a query. The alignment was done using ClustalW (33) and BioEdit (34). Homologous sequences are in the order of sequence identity to the *T. thermophilus* dodecin. Sequences with ≥ 90% pairwise sequence similarity were excluded. Residues involved in FMN binding are marked in orange (deep orange: polar interactions; light orange, hydrophobic interactions), amino acids involved in CoA binding are colored in blue (deep blue, polar interactions; light blue, hydrophobic interactions).
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Due to the increased tilt between the two isoalloxazines, stabilization of the flavin dimer by intermolecular hydrogen bonding is required to present mostly acidic residues on the outer surface of the halophilic protein. The reason for their absence might reside in the requirement to present mostly acidic residues on the outer surface of the halophilic protein (28), which is stable under conditions of >4 M NaCl. Whereas the dodecin from T. thermophilus comprises almost equal amounts of acidic (17%) and basic (17%) residues and exhibits a nearly neutral pl of 5.8, the H. salinarum dodecin exhibits a large excess of aspartates and glutamates (24%) over basic residues (6%) and possesses a remarkably low pl of 3.8. Accordingly, 13 of these acidic residues are localized on the outer surface of the H. salinarum dodecin complex. This does not only provide the required stabilization of the hydrate shell to avoid aggregation by a salting out effect but would also weaken the recognition of negatively charged flavin species by the H. salinarum dodecin (8).

The Binding Site for Trimeric Coenzyme A—Due to the 23 point symmetry of the dodecin complex, 2 kinds of 3-fold symmetric faces can be distinguished; in face I the 3-stranded β-sheet of each monomer extends to a 5-stranded antiparallel β-sheet (Phe-39—Val-41) by local pairing with the β2 strand (Ile-44—Gly-46) of the other monomer. In the 3-fold symmetric face II the β-sheets are continued throughout the whole dodecamer by main chain—main chain pairing between Gly-2—Lys-7 and Glu-9—Thr-13.

The high resolution data for the R65A mutant displayed additional electron density in the 3-fold symmetry face II which could be identified as trimerized coenzyme A molecules (Fig. 9A). As becomes obvious from inspection of the electrostatic surface potential (Fig. 9B), the CoA binding pocket is formed primarily by positively charged (Arg-28, Thr-32, Arg-34, Glu-67) and hydrophobic (Leu-10, Ala-21, Leu-33, Phe-64, and Leu-66) residues. Overall, 3 of the 12 positively charged amino acids per monomer participate in CoA binding.

The thiol group of CoA, which is often acylated in other proteins, is buried in a mainly hydrophobic pocket built by residues Leu-10, Thr-32, Ala-29, and Arg-28 (Fig. 9C). The adenine moiety of the CoA is stabilized by hydrophobic and polar interactions. Besides the stabilization by the perpendicularly arranged residue Phe-64, Leu-33 and Leu-66 form a hydrophobic binding pocket. Aside from hydrophobic interactions, the main chain amide group of Glu-67 and Arg-34 form hydrogen bonds to the adenine atoms N1 and N7, respectively, and the main chain carbonyl groups of Arg-34 and Ala-65 stabilize the adenine N6 atom.

The ribose 3'-phosphate, the 5'-phosphate ester, and the pantetheine primarily form hydrogen bonds and salt bridges. For example, Lys-6 located on the bottom of the binding pocket forms a hydrogen bond with the carbonyl group of the pantetheine. The diphosphate of CoA is bound by salt bridges to residues Thr-32 and Arg-28 and the ribose 2'-hydroxy group H-bonds to residue Arg-34.
Besides the stabilization by the protein environment, the formation of an intermolecular hydrogen bond between the phosphate ester and the hydroxyl group of the pantetheine of the neighboring CoA monomer takes place. Induced by the form of the binding pocket, intramolecular hydrogen bonds between the phosphate ester oxygen atoms (PO10-AO2), the carbonyl group of the pantetheine with the phosphate ester (AO6-AO2), and the 2'-hydroxy group (AO2*) of the ribose with the 3'-phosphate group (AO8, AO9) of the ribose occur, leading to the V-shaped form of the CoA molecules. In contrast to the adenine moiety, the phosphopantetheine, and the thiol group, which are stabilized by a number of amino acids, the 3'-phosphate group of the ribose projects out of the binding pocket, thus making no interactions with the CoA binding site.

From the protein sequence it might not be excluded that the halophilic dodecins are also capable of binding coenzyme A. The archaeal dodecin sequences contain some of the residues of the CoA binding site (Lys-6, Arg-28, Thr-32, Lys-33, Phe-64, Leu-66). However, Arg-34, one of the major constituents of the CoA binding site, is substituted by the negatively charged Asp-34, possibly due to the halophilic characteristics of enhancing the solubility and preventing aggregation in hypersaline solution.

Concluding Remarks—Although the T. thermophilus and the H. salinarum dodecins share the same protein fold and quaternary structure, they differ in their flavin binding characteristics. For the si-si orientation of the FMN dimers in the T. thermophilus dodecin versus re-re orientation in the H. salinarum dodecin, neither the Arg-45 nor the Arg-65 residue alone is responsible as could be expected from the alignment of various dodecin orthologs (Fig. 7). Because most of the residues involved in FMN binding are present in the majority of eu-bacteria (e.g. Tyr-5, Asp-37, Arg-45, Arg-65), the flavin binding mode realized in the T. thermophilus dodecin seems to be generally widespread. The equivalent residues Phe-5, Gly-45, Glu-65 are highly conserved in all archaeal dodecins known so far, indicating that the binding mode exhibited by the H. salinarum dodecin is the prevalent one in Archaea.

The high binding affinity for lumiflavin and lumichrome in the halophilic dodecin led to the hypothesis that lumichrome is the cognate ligand for dodecins in vivo. Accordingly, it was postulated that the dodecins might serve as a waste-trapping device, protecting the cellular environment from high amounts of phototoxic lumichromes, which are generated by the photo-induced degradation of riboflavin (9). In contrast to the H. salinarum dodecin, the T. thermophilus dodecin binds all flavins with similar binding constants. However, this finding is not necessarily contradictory to a supposed function of dodecins as protection against phototoxic compounds because free riboflavin, FMN, or FAD are known to act as photosensitizers with detrimental effects in vivo (29, 30). A scenario for the biological function of dodecins might be that of a flavin trap that gets into action when the cytosolic concentration of free flavin increases, e.g. after heat shock and flavin release from denatured flavoproteins. Notably, dodecins from the mesophiles M. tuberculosis and Streptomyces coelicolor exhibit a similar thermal stability as the T. thermophilus dodecin.4 In this case, the observed retarded flavin binding by apododecin may allow maintaining low resting concentrations of free flavins under nonstressed conditions before switching the dodecin to an efficient flavin scavenger.

Besides the flavin dimers, the T. thermophilus crystal structure also shows the binding of coenzyme A in a trimeric form. Accordingly, dodecins comprise cofactor binding motifs for both pathway-related cofactors, e.g. both cofactors are involved in the synthesis of fatty acids, which are bound in an inert manner either for cofactor storage or to overcome cellular stress caused by transient high cofactor concentrations.

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