Crystalllographic and kinetic analyses of the FdsBG subcomplex of the cytosolic formate dehydrogenase FdsABG from Cupriavidus necator

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Running title: Characterization of FdsBG complex

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Formate oxidation to carbon dioxide is a key reaction in one-carbon compound metabolism, and its reverse reaction represents the first step in carbon assimilation in the acetogenic and methanogenic branches of many anaerobic organisms. The molybdenum-containing dehydrogenase FdsABG is a soluble NAD+-dependent formate dehydrogenase and a member of the NADH dehydrogenase superfamily. Here, we present the first structure of the FdsBG subcomplex of the cytosolic FdsABG formate dehydrogenase from the hydrogen-oxidizing bacterium Cupriavidus necator H16 both with and without bound NADH. The structures revealed that the two iron–sulfur clusters, Fe$_2$S$_4$ in FdsB and Fe$_2$S$_2$ in FdsG, are closer to the FMN than they are in other NADH dehydrogenases. Rapid kinetic studies and EPR measurements of rapid freeze-quenched samples of the NADH reduction of FdsBG identified a neutral flavin semiquinone, FMNH$^+$, not previously been observed to participate in NADH-mediated reduction of the FdsABG holoenzyme. We found that this semiquinone forms through the transfer of one electron from the fully reduced FMNH$^-$, initially formed via NADH-mediated reduction, to the Fe$_2$S$_2$ cluster. This Fe$_2$S$_2$ cluster is not part of the on-path chain of iron–sulfur clusters connecting the FMN of FdsB with the active-site molybdenum-center of FdsA. According to the NADH-bound structure, the nicotinamide ring stacks onto the $re$-face of the FMN. However, NADH binding significantly reduced the electron density for the isosalloazine ring of FMN and induced a conformational change in residues of the FMN-binding pocket that display peptide-bond flipping upon NAD$^+$ binding in proper NADH dehydrogenases.

The oxidation of formate to carbon dioxide is a key reaction in the metabolism of one-carbon compounds. In many anaerobic organisms, the reverse reaction represents the first step in carbon assimilation in both the acetogenic and methanogenic branches of the Wood-Ljungdahl pathway. The genome of Cupriavidus necator H16 (formerly known as Ralstonia eutropha) (1,2) encodes four putative formate dehydrogenases, of which two are soluble NAD$^+$-dependent formate dehydrogenases. One of them is the molybdenum-containing dehydrogenase, FdsABG.

Formate dehydrogenase FdsABG forms a dimer of heterotrimers, $(aβγ)_2$; with the 105 kDa FdsA subunit containing the active site molybdenum center and five iron-sulfur clusters, one Fe$_2$S$_2$ and four Fe$_2$S$_4$ clusters; the 55 kDa FdsB containing a Fe$_3$S$_4$ cluster, FMN, and a binding site for NADH/NAD$^+$; and finally, the 19 kDa FdsG subunit containing one Fe$_2$S$_2$ cluster. Although the physiological function of FdsABG is to oxidize formate to CO$_2$ by reducing NAD$^+$ to

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NADH (3-6), the enzyme has been shown to be fully capable of catalyzing the reverse reaction, the reduction of CO₂ to formate, using NADH as an electron source with steady-state kinetic parameters that conform to the required Haldane relationship (7).

Formate dehydrogenase FdsABG is a member of the NADH dehydrogenase superfamily, which is one of the most broadly distributed families of enzymes (5,8-10). Members of this family typically couple electron transfer to generate a transmembrane proton or sodium gradient. The water solubility of FdsABG formate dehydrogenase greatly simplifies its biochemical study. While the protein does not pump cations, it does contain redox-active centers that have been lost in other members of the superfamily over the course of evolution, including a bis(enedithiolate) molybdenum center that is the site of the fully reversible oxidation of formate to CO₂. As such FdsABG is thought to represent, along with a separate hydrogenase and membrane-terminal domains, an evolutionarily primitive, functional module of NADH dehydrogenases.

The FdsA, FdsB, and FdsG subunits have strong sequence homology to corresponding subunits in the cytosol-exposed arm of the membrane-integral and proton-pumping NADH dehydrogenase (3-6). FdsA is cognate to the Nqo3 subunit of the *Thermus thermophilus* NADH dehydrogenase (11,12), despite the absence of the molybdenum center and of one of the Fe₄S₄ clusters (5). The close homology between FdsA and Nqo3 extends to the presence of a histidine ligand to one of the Fe₄S₄ clusters. The C-terminus of FdsA, containing the molybdenum center, also has an ~60% sequence similarity to the structurally characterized FdhF formate dehydrogenase of *E. coli*, with cysteine, C378, equivalent to the molybdenum-coordinating selenocysteine, U140, in FdhF (13). This C-terminal domain is also present in Nqo3, although the molybdenum center has been lost over the course of evolution.

The FdsB subunit has ~45% sequence identity to the Nqo1 subunit of the *T. thermophilus* NADH dehydrogenase, and like Nqo1 contains a Fe₄S₄ cluster, FMN, and a binding site for NADH/NAD⁺. As demonstrated here, it also possesses an N-terminal thioredoxin-like domain that lacks the typical iron-sulfur cluster and the disulfide bond of thioredoxin. The FdsG subunit has ~34% sequence identity to the Nqo2 subunit of the *T. thermophilus* NADH dehydrogenase and possesses a Fe₃S₂ cluster.

Under physiological conditions, electrons enter the FdsABG holoenzyme at the molybdenum center in the reductive half of the catalytic cycle and depart at the FMN in the oxidative half-reaction, with electron transfer between the two sites (thought to be separated by ~55 Å (9)) mediated by intervening iron-sulfur clusters.

Because FdsB- and FdsG-like domains are found in all members of the NADH dehydrogenase superfamily (12,14), we have undertaken a study of the FdsBG subcomplex of *C. necator* formate dehydrogenase, with the aim of putting the oxidative half-reaction of the enzyme in a structural context. Here, we present the structure of FdsBG complex and characterize its kinetic properties.

**Results**

Previously, the FdsBG homologous, stand-alone complex of NADH dehydrogenase from *Paracoccus denitrificans* has been characterized and shown to be capable of NADH oxidation (15). We therefore set out to characterize the electron transfer reaction of the FdsBG complex from *C. necator*. The recombinant expression of *C. necator* FdsABG holoenzyme in *E. coli* not only produces monomeric and dimeric holoenzyme, but also significant amounts of FdsBG complex (16). A similar observation was reported for the expression of *Rhodobacter capsulatus* FdsG holoenzyme (17).

To streamline the purification of FdsBG complex, we redesigned the FdsABG holoenzyme expression system to produce only FdsBG complex with two different N-terminal tags on FdsG, 6xHis-linker-6xHis- and Twin-Strep®-tag. The resulting constructs produce FdsBG holoenzymes that are substantially identical in their NADH/NAD⁺ redox activity and their ability to crystallize. A detailed description of the two constructs and the purification of the overexpressed FdsBG complex is provided in the ‘Experimental Procedure’ section.

**Characterization of the two iron-sulfur clusters of the FdsBG complex**
We first examined the EPR signals attributable to the complex’s two iron-sulfur centers. Upon extended incubation of FdsBG complex with dithionite, two signals are observed (Fig. 1A, black trace).

The first is seen at liquid nitrogen temperatures with g-values of \( g_{1,2,3} = 2.000, 1.948, \) and 1.920 and linewidths of 1.4, 1.7, and 1.6 mT, respectively. These values are in good agreement with those previously described for the cluster designated as Fe/S\(_1\) for the FdsABG holoenzyme (Fig. 1B, blue dashed trace) (6). This EPR signal is observed well into the 200 K regime.

The second signal is detected below 20 K with g-values of \( g_{1,2,3} = 2.039, 1.955, \) and 1.891 and linewidths of 4.5, 1.4, and 5.3 mT, respectively (Fig. 1B, red dashed trace). This cluster has not been previously described in the holoenzyme (6) and we now designate it as Fe/S\(_2\).

In the homologous FdsBG complexes from NADH dehydrogenase (Aquifex aeolicus NuoEF or Thermoanaerobacterium thermophilus NuoO1-2), only EPR signals of the Fe\(_2\)S\(_2\) clusters are detectable above 77 K, while those for the Fe\(_3\)S\(_2\) clusters appear only below 50 K (18). The g-values \( g_{1,2,3} = 2.004, 1.945, \) and 1.917 for the N\(_{1a}\) Fe\(_2\)S\(_2\) cluster of the 24 kDa subunit of respiratory complex I from bovine mitochondria (the homolog to FdsG) are in excellent agreement with the Fe/S\(_1\) signal. Accordingly, we assign the Fe/S\(_1\) signal to the Fe\(_2\)S\(_2\) cluster of FdsG (19). The signal assigned to the N3 Fe\(_3\)S\(_2\) cluster in the NADH dehydrogenase system, with g-values of \( g_{1,2,3} = 2.037, 1.945, \) and 1.852, is also in good agreement with the Fe/S\(_3\) signal of the FdsBG complex. Therefore, we assign the Fe/S\(_3\) signal to the Fe\(_3\)S\(_4\) cluster of FdsB.

We had initially assigned the Fe/S\(_1\) signal to the His-coordinated Fe\(_3\)S\(_4\) cluster contained in the FdsA subunit of the FdsABG holoenzyme (6), but that is clearly not the case as the signal is observed in the FdsBG complex. We note that the Fe/S\(_3\) signal has very similar g-values to that reported for the previously observed Fe/S\(_3\), although the Fe/S\(_3\) linewidths are considerably broader (see the g\(_1\) and g\(_3\) features of red and blue EPR spectra, centered around the dashed lines in Fig. 1C). However, the Fe/S\(_3\) cluster has been shown to be magnetically coupled to the molybdenum center and must accordingly arise from the Fe\(_3\)S\(_4\) cluster proximal to the molybdenum center in FdsA. Therefore, the Fe/S\(_3\) signal must be a newly identified signal.

The assignment of the Fe/S\(_3\) signal to the Fe\(_3\)S\(_4\) cluster of the FdsB subunit is strengthened by the below described structure of the FdsBG complex and by our previously published structural model of the FdsABG holoenzyme (9). This model places the Fe\(_3\)S\(_4\) cluster in the proximity of FdsA in the intact holoenzyme and in the electron path between the molybdenum and FMN. It is likely that the large linewidths, in conjunction with the relatively low level of reduction and overlapping g-values with Fe/S\(_3\), obscured its detection in the earlier work with the holoenzyme. It is also possible that the presence of additional, high-potential clusters in the FdsA subunit prevents the reduction of the Fe/S\(_3\) cluster in the holoenzyme.

**Crystal structure of FdsBG complex – overview**

We further characterized the FdsBG complex by determining its structure by X-ray crystallography to 2.3 Å resolution (Fig. 2B-2E). The final phased electron density allowed us to build nearly all residues of the FdsBG heterodimer (for data and refinement statistics see Table S1). The heterodimer has the dimensions of 82 Å x 64 Å x 62 Å and the two heterodimers of the asymmetric unit are related by a non-crystallographic rotation of ~179° (Fig. 2A). The axis of rotation passes through the interface formed by the N-terminal thioredoxin-like domains of the two FdsB molecules of the asymmetric unit (residues 3\(^B\) to 104\(^B\) - superscript B indicates that the residue belongs to FdsB). The unusual relationship of the two FdsBG complexes in the asymmetric unit notwithstanding, their structures are nearly identical with an RMSD of 0.35 Å for all the atoms of the 661 residues of the core of the 735-amino acid FdsBG complex. Each FdsBG complex contains two iron-sulfur clusters (one Fe\(_2\)S\(_2\) and one Fe\(_3\)S\(_4\) cluster), one FMN, and one K\(^+\) ion. The final model also contains 498 water molecules.

For the detailed analysis of the structural differences of FdsBG complex to its homologous subcomplex from NADH dehydrogenase, we used the recently published, high-resolution structure from A. aeolicus, NuoEF complex (20). The NuoE subunit is homologous to the Nuo1 and Fds subunits, and the NuoF subunit is...
homologous to the Nqo2 and FdsB subunits (Fig 3A and 3B).

**Structure of the FdsB subunit of the FdsBG complex**

Except for the N-terminal thioredoxin-like domain (residues 3^B^ to 104^B^), the FdsB subunit structure is highly similar to that of the NuoF subunit of the *A. aeolicus* NADH dehydrogenase (RMSD of 1.48 Å for 394 Ca atoms). Like NuoF, FdsB contains a Rossmann-like fold (residues 138^B^-322^B^, also classified as Complex I_51K domain (21)), a ubiquitin-like domain (residues 338^B^-423^B^), and a four-helical bundle (residues 426^B^-511^B^, NADH_4Fe-4S domain). The Rossmann-like fold contains the FMN binding site and the C-terminal four-helical bundle contains the Fe\(S\)\(_4\) cluster (22) (Fig. 3A).

In the Rossmann-like domain, the main structural differences between the FdsB and NuoF subunits are in loops that are solvent-exposed. The only loop with a structural difference that is buried in the complex, is positioned between the bound FMN and the Fe\(S\)\(_2\) cluster of the FdsG subunit (183^B^-190^B^ loop), thus enabling the Fe\(S\)\(_2\) cluster to be ~0.2 Å closer to the FMN in the FdsBG complex than in the NuoEF complex (Fig. 4A).

The ubiquitin-like domain of FdsB is tilted towards the FMN bound in the Rossmann-like domain. This tilt towards the FMN propagates into the four-helical bundle domain, resulting in an ~0.2 Å distance closer between the FMN and the Fe\(S\)\(_4\) cluster compared to that seen in the NuoEF complex (Fig. 4B). The Fe\(S\)\(_4\) cluster is located in a hydrophobic environment close to the surface and is coordinated by C443^B^, C446^B^, C449^B^, and C489^B^ (Fig. 2E).

The N-terminal thioredoxin-like domain of FdsB is structurally similar to the C-terminal thioredoxin-like domain of FdsG (RMSD 1.97 Å for 71 Ca atoms of the core of the domain). However, unlike the domain in FdsG, that in FdsB has lost its capacity to bind a Fe\(S\)\(_2\) cluster due to mutation of the corresponding cysteines to proline, alanine, serine, and phenylalanine, respectively (P10^B^, A15^B^, S45^B^, and F49^B^).

**Structure of the FdsG subunit of the FdsBG complex**

The structure of the FdsG subunit is also highly similar to the NuoE subunit of *T. thermophilus* NADH dehydrogenase. Like NuoE, FdsG consists of an N-terminal four-helical bundle (residues 29^G^-74^G^) and a C-terminal thioredoxin-like domain (residues 79^G^-159^G^). The domains are separated by a four-amino acid long linker, around which the two domains are hinged by ~26° compared to NuoE. This domain arrangement is stabilized by a K\(^+\) ion, which is held in place by residues from both domains (H71^G^, H112^G^ and E123^G^). Fig. 4C.

Although the N-terminal domains of FdsG and NuoE consist of a four-helical bundle, the first helix of FdsG packs in parallel to the second and third helix, while in NuoE it runs across the interface formed by these two helices (Fig. 4C). The C-terminal thioredoxin-like domain of FdsG is structurally similar to the N-terminal thioredoxin-like domain of FdsB but contains a spinach ferredoxin-like Fe\(S\)\(_2\) cluster (23). The Fe\(S\)\(_2\) cluster is located in a hydrophobic environment close to the surface, and is coordinated by C86^G^, C91^G^, C127^G^, and C131^G^ (Fig. 2D and 3B). In comparison to Nuo2, FdsG’s and NuoE’s thioredoxin-like domains are truncated at their C-termi and are missing a disulfide bond (Fig. 3B).

**The interface between FdsB and FdsG subunits in the FdsBG complex**

Two helices of FdsG’s four-helical bundle interact with the surface of the Rossmann-like domain of FdsB, similar to the interactions seen in the NuoEF interface (Fig. 4D). The C-terminal thioredoxin-like domain of FdsG binds to the same surfaces of the ubiquitin-like domain and Rossmann-like domain of FdsB as in the NuoEF complex. However, the different positioning of the ubiquitin and of the 183^B^-190^B^ loop results in the Fe\(S\)\(_2\) cluster of FdsG being an additional ~1.0 Å farther from the Fe\(S\)\(_4\) cluster compared to the respective clusters in the NuoEF complex (Fig. 4E and 4F).

With an edge-to-edge distance of nearly 21 Å, direct electron transfer between the Fe\(S\)\(_2\) cluster and Fe\(S\)\(_4\) cluster is unlikely to be kinetically significant. However, both clusters are within 12 Å of the isoalloxazine ring of FMN (Fig. 4E), which evidently mediates electron equilibration between the two iron-sulfur clusters. While the
Fe₄S₄ cluster of FdsB is clearly a part of the electron transport chain between the molybdenum center of FdsA and the FMN of FdsB, the Fe₃S₂ cluster of FdsG lies off-path and appears to function as a temporary repository for electrons in the course of electron egress out of the flavin (see ‘Discussion’ section).

The FMN binding site of the FdsB subunit

The FMN is bound by a network of interactions in an extended, solvent-accessible cavity that also accommodates NAD⁺/NADH. The isoalloxazine ring of FMN is within 12 Å of each iron-sulfur cluster. The C8-methyl of the dimethylbenzene moiety of the flavin points towards the Fe₄S₄ cluster and the N5-containing edge faces the Fe₃S₂ cluster, while the ribityl phosphate tail extends away from both iron-sulfur clusters (Fig. 4E).

The direct and water-mediated interactions between the FMN isoalloxazine ring and the ribityl phosphate tail with FdsB’s Rossmann-like fold are conserved in the high-resolution structures of other members of the NADH oxidoreductase family (14,20). (Fig. 5A-5C. For a detailed description and the 2D representation of these interactions see supporting material and supporting Fig. S1, respectively. For comparison, we also provide the 2D representation of the FMN interactions with the NADH dehydrogenase, NuoE and the NAD⁺ reducing hydrogenase, HoxF as supporting Fig. S2 and S3, respectively. These 2D representations of the interactions were prepared with LigPlot+ v.2.1. (23).)

Biochemical characterization of the bound FMN

In the absence of the FdsA subunit, which contains five iron-sulfur clusters and a molybdenum active site, the UV-visible spectrum of the isolated FdsBG complex is dominated by the spectral features of the FMN centered around 350 nm and 450 nm (Fig. 6A, red spectrum). In addition, a feature around 550 nm, attributed to the Fe₄S₄ and Fe₃S₂ clusters of the FdsBG complex, is significantly more pronounced due to the lack of interference of the additional iron-sulfur clusters present in the FdsABG holoenzyme (6). Removal of the FMN via TCA precipitation indicates that the FdsBG complex is approximately 50% saturated with FMN. This level of FMN saturation is comparable to the level of flavin saturation in the isolated FdsABG holoenzyme.¹

This incomplete FMN saturation in FdsBG, suggests that we are visualizing in our crystal structures an average of at least two kinds of FdsBG, one with and one without FMN bound, deflavo FdsBG.

A reductive titration with sodium dithionite results in a systematic bleaching of the absorbance throughout the UV-visible region (Fig. 6A, blue spectrum). At the end of the titration, the bleaching in the 550 nm region is incomplete, suggesting that the cluster principally responsible for the residual 550 nm absorption is the Fe₄S₄ cluster that is not fully reduced by dithionite. A plot of the fractional absorbance changes at 550 nm against that at 450 nm provides information about the relative order of reduction during titration. The changes at 550 nm and at 450 nm are dominated by the reduction of the iron-sulfur clusters and FMN, respectively. A concurrent reduction of iron-sulfur clusters and FMN will result in a diagonal in this plot. A deflection of the diagonal to the upper left suggests an earlier reduction of iron-sulfur clusters while to the lower right suggests an earlier reduction of FMN (24). The inset of Fig. 6B, shows a deflection to the upper left from the diagonal, indicating that at least one iron-sulfur cluster is reduced sooner than the FMN during titration with dithionite. This suggests that the FMN has a lower reduction potential relative to at least one of the iron-sulfur clusters.

NADH binding to the FdsB subunit - overview

To establish the NADH/NAD⁺ binding site of the FdsBG complex, we solved the structure of FdsBG with bound NADH. To this end, preformed and stabilized FdsBG crystals were soaked with NADH at a final concentration of 10 mM in stabilization solution for two hours, before flash freezing in liquid nitrogen. The crystal structure was solved to 2.0 Å by molecular replacement using the above described structure of the FdsBG complex as the initial search model. (See Table S1 for diffraction and refinement

¹ D. Niks and R. Hille, unpublished data
Electron density for the adenosine diphosphate moiety of NADH is visible in the unbiased (Fo-Fc)-map of both FdsBG complexes of the asymmetric unit (Fig. 7A, green mesh). The overall protein structures of the FdsBG complexes are virtually identical in the presence and absence of NADH (RMSD 0.19 Å for the 1435 Ca atoms shared between the FdsBG complex on its own and NADH-bound structures).

As in the FdsBG structure by itself, the two NADH-bound FdsBG complexes of the asymmetric unit are highly similar (RMSD 0.34 Å for the 660 Ca atoms that the two FdsBG complexes share). However, the density for the isoalloxazine ring of FMN in each of two FdsBG complexes of the asymmetric unit is distinct. In one FdsBG complex most of the FMN has significantly lower electron density than its terminal phosphate (Fig. 7A, light blue versus blue meshes). Due to our crystallization conditions, we speculate that the terminal phosphate is actually a sulfate bound to the fraction of FdsBG with no FMN bound.

FMN-binding to FdsBG in the presence of NADH

Comparing the FMN binding pocket of both FdsBG complex in the asymmetric unit, suggests that the peptidyl bond between D184B and E185B is pointing towards the O4 carbonyl oxygen of the isoalloxazine ring of FMN, in particular when less FMN is bound. The refinement of the FdsBG structure without FMN places the carbonyl oxygen of the D184B-E185B peptide bond so far into the FMN binding pocket that it would encroach on the O4 of the isoalloxazine ring (Fig. 7B). This suggests that the change of the peptide bond position correlates with the loss of FMN.

NADH/NAD+ binding site on FdsBG complex - the adenosine diphosphate moiety

More electron density is visible for the adenosine diphosphate of NADH than for the nicotinamide portion. This observation implies that the nicotinamide moiety of NADH contributes less to NADH affinity to FdsBG.

The β phosphate of NADH is held in place by a salt bridge to FdsB and by two hydrogen bonds to the O2' and O4' hydroxyl groups of FMN’s ribityl moiety (Fig. 7D). The adenosine base of NADH binds into a hydrophobic pocket. The O2' hydroxyl and O3' hydroxyl adenosine are in hydrogen bond distance to the carboxyl group of E275B. This places a negative charge adjacent to the O2', ensuring FdsBG's specificity for NAD+/NADH over NADP+/NADPH (25). (A more detailed description of the ADP binding interactions with FdsB is provided in supporting material.)

The specificity for the adenine base is brought about by the hydrogen bond from the side chain hydroxyl group of T168B which is specific to FdsB. In the NADH dehydrogenases and NAD+ reducing hydrogenases, the threonine is replaced by either phenylalanine or leucine. This hydrogen bond interaction between the adenine base and threonine contributes to the more than 0.5 Å distant binding of the adenosine diphosphate relative to the FMN isoalloxazine ring compared to its binding in NuoF (Fig. 7C).

Binding of the nicotinamide ring of NADH relative to the isoalloxazine ring of FMN

Although only weak electron density is evident for the nicotinamide ring of NADH in the structure of both FdsBG complexes of the asymmetric unit, the direction of the two phosphates visible in the electron density suggests that the nicotinamide is projected toward the solvent exposed re-face of the isoalloxazine ring of FMN. We therefore suggest that the nicotinamide moiety of NAD+/NADH stacks onto the re-face of the isoalloxazine ring during the actual electron transfer step, similar to what is observed in the NuoEF complex with bound NAD+ and NADH (Fig. 7C; (20)). However, the more distant binding of the adenosine relative to the isoalloxazine ring, suggests that the nicotinamide ring will also be displaced over the isoalloxazine ring during the electron transfer reaction as compared to that in NuoF.

Rapid-reaction kinetics of the electron transfer reaction of the FdsBG complex

Due to the high velocity of the electron transfer reaction, we performed the rapid-reaction kinetics of FdsBG reduction by NADH at 5 °C. Although this is the reverse of the physiological reaction for the formate dehydrogenase, it is the physiological
direction seen in NADH dehydrogenase and most other members of this enzyme family. As illustrated by a typical kinetic trace in the inset of Fig. 8, the reaction is biphasic. The fast phase of the reaction is complete within 120 ms. A plot of the observed rate constant for the fast phase as a function of NADH concentration is hyperbolic (Fig. 8), yielding a limiting $k_{\text{red}}$ of 680 s$^{-1}$ at high concentration of NADH, and a $K_d^{\text{NADH}}$ of 190 μM. Assuming a doubling of the rate constant for every 10 °C increase in temperature, this corresponds to a limiting $k_{\text{red}}$ of 3850 s$^{-1}$ at 30 °C, a rate that is more than six-fold faster than the limiting rate of reduction of FdsABG holoenzyme at high concentration of formate, some twenty-fold faster than the $k_{\text{cat}}$ of the FdsABG holoenzyme for formate oxidation (6), and 350-fold faster than the $k_{\text{cat}}$ for CO$_2$ reduction (7).

Formulation of a neutral FMN$\bullet$ semiquinone intermediate in the course of the reaction of FdsBG complex with NADH

At the completion of the fast phase of the reaction of 10 μM FdsBG complex with 5 μM NADH, the transiently observed UV-visible spectrum exhibits absorption in the 500 - 550 nm region consistent with the appearance of a neutral flavin semiquinone (FMN$\bullet$) (Fig. 9A, red 0.3 s spectrum). This species, not seen with the FdsABG holoenzyme, is the result of transfer of a single electron from the fully reduced flavin hydroquinone formed in the initial reaction with NADH to one or the other of the iron-sulfur clusters, leaving an FMN$\bullet$ semiquinone behind; as described further below, this iron-sulfur cluster is the Fe$_2$S$_2$ cluster of FdsG giving rise to the Fe/S$_1$ EPR signal (see also Scheme 1).

On a longer timescale, the system disproportionates to some degree in a second-order process (Fig. 9A, blue 10 s spectrum), with an electron passing between two equivalents of FdsBG$_{2e}$ to give one equivalent each of FdsBG$_{1e}$ and FdsBG$_{3e}$. The FdsBG$_{1e}$ will have its Fe$_2$S$_2$ cluster (Fe/S$_1$) reduced and its FMN oxidized, and FdsBG$_{3e}$ will have both Fe/S$_1$ and the FMN reduced. The reaction shown in Fig. 9A was carried out with stoichiometric NADH so that the FdsBG$_{1e}$ formed will not be further reduced by reaction with a second equivalent of NADH. The inset of Fig. 9A displays the kinetic time course obtained at 450 nm (extracted from the full dataset collected with a photodiode array detector), showing both the fast reduction and the much slower disproportionation reactions.

We note that FdsBG disproportionation may also involve the reaction of FdsBG$_{3e}$ with the deflavo form of FdsBG complex (which accounts for approximately 50% of the total protein as described above). While the deflavo form cannot be reduced by NADH due to the absence of the FMN, it can accept electrons from the fully constituted and reduced FdsBG$_{2e}$, resulting in one equivalent of FdsBG$_{1e}$ with an oxidized FMN and reduced Fe/S$_1$ and a deflavo FdsBG$_{1e}$ containing only a reduced Fe/S$_1$.

As the two processes are expected to proceed on at least approximately the same time scale, at the end of disproportionation there will be an excess of enzyme containing more oxidized than reduced flavin. An analysis of the slow phase of the reaction (described in the ‘Experimental section’) yields a second-order rate constant for this disproportionation on the order of $10^3$ M$^{-1}$s$^{-1}$. It is noteworthy that, particularly at higher NADH concentrations, the amplitude of the disproportionation phase decreases as the oxidized FMN thus generated becomes re-reduced by reaction with a second equivalent of NADH (data not shown).

Characterization of the neutral semiquinone intermediate, FMN$\bullet$

The spectral signature of the FMN$\bullet$, which has not been previously characterized in the FdsABG holoenzyme, can be best observed in the difference spectra presented in Fig. 9B. At the completion of the fast phase described by the absorbance difference between the spectrum of the fully oxidized enzyme subtracted from the spectrum collected at 0.3 s after the initial reaction of FdsBG with NADH (black difference spectrum). A positive feature consistent with the formation of the FMN$\bullet$ is observed in the 500 – 650 nm region, although attenuated by the decrease in the absorbance due to the concurrent reduction of the iron-sulfur clusters. The complex negative feature in the 300 – 500 nm region is dominated by the loss of absorbance associated with iron-sulfur reduction as well.

The disappearance of the FMN$\bullet$ is also evident in the disproportionation phase of the reaction (and is even more distinct, being unaffected by
any further reduction of the iron-sulfur clusters). In the difference spectrum obtained by subtracting the spectrum collected at 0.3 s from that at 10 s after initial reaction with NADH (red difference spectrum), the 500–650 nm region is defined by two broad peaks centered at 570 and 605 nm, characteristic for the presence of FMNH• (26). The features are negative as they reflect the disappearance of the FMNH•.

Further evidence for the presence of a neutral semiquinone intermediate FMNH• comes from EPR measurements. To capture the transiently formed FMNH•, the enzyme was reacted with NADH at 0 °C and rapid freeze quenched (RFQ) with a quenching time of ~40 ms. Fig. 10A shows the resulting spectrum collected at 150 K.

The 150 K EPR spectrum includes contributions from both FMNH• and Fe/S1, demonstrating that it is the Fe2S2 cluster of FdsG that has been reduced. Graphical subtraction of the Fe/S1 signal from the measured EPR spectrum yields an isotropic EPR signal with a giso = 2.003 and a linewidth of ~1.9 mT which is indicative of a neutral rather than anionic semiquinone (27,28). Integration of both, the scaled Fe/S1 spectrum and the FMNH• spectrum from the graphical subtraction yielded a spin density ratio Fe/S1:FMNH• of 1:0.7 consistent with the formation of FMNH• and reduction of only a single iron-sulfur cluster at the end of the fast phase of the NADH reduction of the FdsBG complex. No additional iron-sulfur signal was seen at 9 K either (data not shown).

To ascertain whether reduction of the Fe/S1 cluster occurred during disproportionation, an additional sample was prepared by incubating FdsBG complex with an excess of NADH for 10 s under anaerobic conditions. The resulting spectrum recorded at 9 K is shown in Fig. 10B. The spectrum illustrates that only the spectrum corresponding to a single iron-sulfur cluster, Fe/S1 is present at the endpoint of the disproportionation phase. Taken together, these results indicate that the fully reduced FMNH2 initially formed on reaction with NADH breaks down by electron transfer of a single electron to the Fe2S2 cluster (Fe/S1), with the second electron remaining as FMNH•; the Fe2S2 cluster (Fe/S3) remains oxidized (see Scheme 1).

Discussion

The genome of C. necator H16 (1,2) encodes four putative formate dehydrogenases, of which two are soluble NAD+-dependent formate dehydrogenases. One of them is a molybdenum-containing dehydrogenase (FdSAG) and the other is a tungsten-containing dehydrogenase (FdwAB) (29). While the FdsB of the molybdenum-containing dehydrogenase has an additional N-terminal thioredoxin-like domain, the FdsB homolog of the tungsten-containing dehydrogenase is N-terminally fused to its FdsG homolog to form FdwB. Two other NADH oxidoreductases with homology to FdsAG and FdwAB are also present in C. necator -- NAD dehydrogenase and NAD+ reducing hydrogenase.

Functional relevance of the N-terminal thioredoxin-like domain of FdsB

The N-terminal thioredoxin-like domain of FdsB has structural similarity to the thioredoxin-like Fe2S2 ferredoxin domain from A. aeolicus (21) (RMSD of 1.67 Å of 89 Ca atoms between both domains). The A. aeolicus domain is known to dimerize. The largest contact interface between two FdsBG complexes within the asymmetric unit is formed between the two thioredoxin-like domains. This suggests that the thioredoxin-like domain of FdsB contributes to the dimerization of the FdsAG holoenzyme.

We suggest, however, because of: a) the relative small size of ~560 Å2 of buried area, b) the presence of only a few interactions across the interface (Fig. S4A - S4D), c) the low conservation of the residues at the dimer interface (Fig. S4E and S4F), and d) the absence of a dimeric form in solution during purification (data not shown), that the dimer formation in the asymmetric unit is merely due to crystal packing (30,31) and does not participate in the dimerization of the FdsAG holoenzyme.

Although the N-terminal thioredoxin-like domain of FdsB is absent in other members of the NADH dehydrogenase family, it may nevertheless contribute to the stability of FdsB within the FdsAG holoenzyme. Protein stability is apparently a concern for some members of the family, as FdwB and HoxF have their FdsG and FdsB homologs fused. The linker between the FdsB-FdsG homologs overlaps structurally with the N-terminal thioredoxin-like domain of FdsB.
The absence of the Fe$_2$S$_2$ cluster in the FdsG homologous region of FdwB and HoxF might destabilize this region to such an extent that its stability is only maintained by the fusion to their FdsB homologous regions.

Finally, we cannot exclude that the N-terminal thioredoxin-like domain provides an interface for an unknown partner. This partner may either participate in the redox reaction or provide a connection to the cell’s metabolic status, such as ACP does in the case of NADH dehydrogenase (32,33).

**Electron transfer mechanism in FdsBG**

In the FdsABG formate dehydrogenase of *C. necator*, the reducing equivalents from the oxidation of formate pass through a chain of iron-sulfur clusters from the molybdenum center in FdsA to the FMN in FdsB, which ultimately reduces NAD$^+$. The FdsBG subcomplex can perform this ultimate step of reducing NAD$^+$, as can the corresponding subcomplexes from both NADH dehydrogenase (15,20,34) and NAD$^+$-reducing hydrogenase (35).

In our FdsBG structure, the Fe$_4$S$_4$ cluster of FdsB is in close proximity to the C8-methyl of FMN and to highly conserved surface residues that are implicated in the interaction of FdsB with FdsA (i.e., E441$^B$-S487$^B$, I451$^B$-G452$^B$, and K292$^B$-L298$^B$). This is consistent with the Fe$_4$S$_4$ cluster being on-path between the FMN and the molybdenum center of the FdsABG holoenzyme. The Fe$_2$S$_2$ cluster of FdsG, on the other hand, is farther away from residues implicated in Fda binding, and is also too far from the Fe$_4$S$_4$ cluster to directly take part in electron transfer from the molybdenum center to the bound FMN.

Nevertheless, it is clear from the evidence presented here that the initial electron transfer event out of the FMN upon reduction by NADH is to the off-path Fe$_2$S$_2$ cluster (having the higher reduction potential) rather than to the on-path Fe$_4$S$_4$ cluster (with much lower potential), as shown in Scheme 1. This process has not been observed previously in the reduction of either the the FdsABG holoenzyme or the NADH dehydrogenase by NADH. It is thought that a similar off-path electron transfer minimizes the formation of reactive oxygen species (ROS) in

NADH dehydrogenase by minimizing the accumulation of neutral flavin semiquinone, FMNH$^+$ (22,36). In the case of FdsBG, the on-path electron transfer from the fully reduced flavin hydroquinone to the Fe$_2$S$_2$ cluster, to the extent that it occurs, leaves a neutral flavin semiquinone, FMNH$. This semiquinone would then rapidly transfer the second electron to the Fe$_2$S$_2$ cluster, thereby minimizing accumulation of the neutral semiquinone in the NADH dehydrogenase.

**Implications for electron transfer in other members of the NADH dehydrogenase superfamily**

Detailed structural analysis of the reduced and oxidized NuoEF complex from *A. aeolicus* revealed that the peptide bond between E95$^F$ and S96$^F$ of NuoF undergoes a reversible flip depending on the oxidation state of the protein (20). In the oxidized state, the carbonyl group of E95$^F$ points to the FMN, while in the reduced state it points to the Fe$_2$S$_2$ cluster (Fig. 11D).

The equivalent peptide bond in the FdsBG complex (E185$^B$-G186$^B$) adopts the same orientation in either state, oxidized and reduced (i.e., FdsBG as isolated and in the presence of NADH, respectively; Fig. 11C). However, in a sub-population of the reduced FdsBG complex, the peptide bond prior to the oxidation state-sensitive peptide bond (D184$^B$ - E185$^B$) is pointing toward the isoalloxazine ring of FMN, even encroaching on its O4 carbonyl oxygen. We have attributed this encroachment to the low FMN occupancy of the NADH-reduced FdsBG.

We note that this region of the protein is not particularly well-conserved between NuoF and FdsB (94$^F$-DESEP-98$^F$ versus 184$^B$-DEGDS-188$^B$, the residues of the peptide which reorient dependent on the oxidation state in NuoEF are in bold. See also Fig. 11B). The equivalent region in HoxF (232$^F$-DEGEP-236$^F$) appears to be flexible as well. As HoxF is devoid of a Fe$_2$S$_2$ cluster (one cysteine coordinating the Fe$_2$S$_2$ cluster is an alanine and another cysteine is a serine), it is more likely that the absence of FMN in the reduced state of the Hox-complex allows this peptide region to undergo the conformational change (14).

These observations highlight the inherent flexibility of this region, but also suggest that the oxidation state-dependent flipping of the peptide
bond may be unique to the proper NADH dehydrogenase, and not shared across the family.

**Conclusion**

In the present work, we have determined the X-ray crystal structure of the FdsBG portion of the FdsABG holoenzyme and characterized its interaction with NADH. The flexible region between the FMN and the Fe₂S₂ cluster in NADH dehydrogenase has recently been reported to undergo a conformational change dependent on the oxidation state in proper NADH dehydrogenases; however, an oxidation state-dependent conformational change is not seen here with the formate dehydrogenase.

In addition, we see for the first time the formation of a neutral semiquinone, FMNH⁺, in the course of reduction by NADH as a result of an electron transfer from the fully reduced flavin hydroquinone to the Fe₂S₂ cluster. The hydroquinone is generated upon reaction with NADH. The electron transfer to the Fe₂S₂ cluster is off-path with respect to the electron transfer pathway to the molybdenum center where the enzyme reduces CO₂ to formate in the FdsABG holoenzyme. The electron transfer to the Fe₂S₂ cluster occurs over a distance of some 12 Å, with a rate constant that must exceed that for the NADH concentration-dependent rate of FMN reduction. The rate of FMN reduction is the rate-limiting step for electron transfer and occurs with a rate of at least 680 s⁻¹ at 5°C. This electron transfer process to the Fe₂S₂ cluster, observed here for the first time, has previously been proposed to play a role in limiting reactive oxygen species (ROS) generation in the case of NADH dehydrogenase.

**Experimental procedures**

*Cloning, protein expression, and protein purification of FdsABG holoenzyme and FdsBG subcomplex*

The fdsGBACD operon with a 6xHis-linker-6xHis-linker fused to the N-terminus of the FdsG subunit was cloned into a pTrcHisB vector to construct the pTrc12HLB-FdsGBACD plasmid as previously described (16).

For protein expression, the plasmid was transformed into *E. coli* DH5α cells. By varying expression conditions, protein synthesis was optimized for production of FdsBG complex at the expense of FdsABG holoenzyme. For the optimized procedure, a 50 ml starter culture of LB media with 100 μg/ml Ampicillin was inoculated from a glycerol stock of the DH5α transformed cells and grown overnight at 37°C. The overnight culture was used to inoculate three 6 L Erlenmeyer flasks each containing 2 L of TB media supplemented with 100 μg/ml Ampicillin and grown at 30°C and 200 rpm to an OD₆₀₀ ~0.4, at which time they were induced with 0.25 – 0.5 mM IPTG. The induced cells were grown for an additional 44 – 48 hours until OD₆₀₀ reached ~10. Harvested cells were frozen in liquid nitrogen and stored at -80°C until further use.

The plasmid pTrc12HLB-FdsGBACD was modified by removing the gene encoding the FdsA subunit, to yield pTrc12HLB-FdsGBCD. The pTrc12HLB-FdsGBCD plasmid was transformed into *E. coli* DH5α cells and the protein FdsBG was expressed using the same procedure as described above. In addition, the plasmid pTrc12HLB-FdsGBCD was further modified by replacing the 6xHis-linker-6xHis-tag for a Twin-Strep-tag® (IBA, Göttingen, Germany; Twin-strep-tag sequence: WSHPQFEKGGGSGGGSGGGSAWSHPQFEKSG) to yield pTrc-strep-FdsGBCD. The construction of this plasmid was outsourced (Epoch Life Science, Sugar Land, TX).

For overexpression of the FdsBG complex, the pTrc-strep-FdsGBCD plasmid was transformed into *E. coli* DH5α cells. A 50 ml starter culture of LB media supplemented with 100 μg/ml Ampicillin was grown overnight at 37°C. The overnight culture was used to inoculate two 6 L Erlenmeyer flasks each containing 2 L of TB media supplemented with 100 μg/ml Ampicillin and grown at 30°C and 200 rpm to an OD₆₀₀ ~0.4, at which time they were induced with 0.25 mM IPTG. The induced cells were grown for an additional 16 hours at 30°C and 160 rpm (final OD₆₀₀ 12-16). Harvested cells were frozen in liquid nitrogen and stored at -80°C until further use.

**Purification of 6xHis-linker-6xHis-tagged FdsBG complex** – All steps were performed on ice or at 4°C. Frozen cells were thawed and suspended in 40 mM K₂PO₄, 10 mM KNO₃, at pH 7.2 in the presence of lysozyme, DNase I, 0.5 mM PMSF, 1 mM benzamidine-HCl, and 1 mM NaF. The
resuspended cells were passed 1-2 times through a French Press at 10-15 kpsi. Cell debris was removed by centrifugation for 45 min at 200,000x g.

For the initial purification, a 20-40% (NH₄)₂SO₄ cut was performed. The collected (NH₄)₂SO₄ cut was dissolved in 40 mM K-PO₄, 10 mM KNO₃, at pH 7.5 containing 15 mM imidazole, 0.5 mM PMSF, 1 mM benzimidine-HCl, and 1 mM NaF. The supernatant was incubated for one hour with Ni-NTA Agarose beads (Qiagen, Germantown, MD). The beads were then transferred into an empty column and washed with five column volumes of resuspension buffer containing 45 mM imidazole. The FdsABG holoenzyme and FdsBG complex were eluted from the column with 300 mM imidazole. Ammonium sulfate was added to the eluent to 50% saturation and the precipitate was collected. To reduce the contact time (~1.5 hours) between FdsBG complex and the Ni-NTA resin, the chromatography was performed with a head pressure of ~2 psi of N₂.

The ammonium sulfate precipitate was treated in one of two ways: For preparations containing only FdsBG complex (the product of the overexpression of pTrc12HLB-FdsGBCD plasmid) no further purification was performed. The precipitate was resuspended, and buffer exchanged into 10 mM HEPES-NaOH, at pH 7.2 with a PD-10 column (GE Healthcare, Pittsburgh, PA) or by ultrafiltration with an Amicon Ultra with a MWCO of 50 kDa (MilliporeSigma, Burlington, MA).

For preparations containing a mixture of FdsABG holoenzyme and FdsBG complex (the product of the overexpression from pTrc12HLB-FdsGBACD plasmid), the ammonium sulfate precipitate was dissolved in resuspension buffer containing 150 mM NaCl. The FdsBG complex was separated from FdsABG holoenzyme by gel filtration (Superdex 200 200 PG, GE Healthcare, Pittsburgh, PA). The concentration and quality of FdsBG complex for each fraction of the gel filtration chromatography was assessed by ratio of the absorption at 280 and 450 nm; pure FdsBG complex has a 280/450 nm ratio of 3.4. Fractions of sufficient purity and quality were combined, concentrated by ultrafiltration and buffer exchanged into 10 mM HEPES-NaOH, pH 7.2 with a PD-10 column or by ultrafiltration with an Amicon Ultra with a MWCO of 50 kDa. The concentrated protein from both preparations was aliquoted, flash-frozen in liquid nitrogen, and stored in liquid nitrogen until further use.

### Purification of Twin-Strep-tagged FdsBG complex

- All steps were performed on ice or at 4 °C. Frozen cells were thawed and resuspended in 40 mM K-PO₄, at pH 7.5 in the presence of lysozyme, DNase I, 0.5 mM PMSF, 1 mM benzimidine-HCl, and 1 mM NaF. The resuspended cells were passed 1-2 times through a French Press at 10-15 kpsi. Cell debris was removed by centrifugation and the clarified supernatant was loaded onto a 10 ml Strep-Tactin® XT Superflow® column (IBA, Gottingen, Germany) with 2.5 cm diameter at 0.5 ml/min. The flow-through was loaded an additional time over the Strep-Tactin® column, before washing the column with 40 column volumes of resuspension buffer. FdsBG complex was eluted with resuspension buffer containing 50 mM biotin. The eluent was concentrated by ultrafiltration and buffer exchanged into 10 mM HEPES-NaOH, pH 7.2 with a PD-10 column or by ultrafiltration using an Amicon Ultra with a MWCO of 50 kDa. The protein was aliquoted, flash-frozen in liquid nitrogen, and stored in liquid nitrogen until further use.

### Structure determination of the FdsBG complex by X-ray crystallography

**Crystallization** – 6xHis-linker-6xHis-tagged FdsBG complex was crystallized under anaerobic conditions by vapor diffusion in a sitting drop format. 3 μl of FdsBG complex at 2.5 mg/ml were mixed with 1 μl of reservoir solution and setup over 500 μl of reservoir solution. Oxygen was removed from all solutions used for crystallization by bubbling ISP-grade nitrogen through them. 5 mM DTT was added to the FdsBG stock solution prior to the removal of the oxygen. Crystals for native and anomalous data collection were grown over 5-6.5% [v/v] isopropanol, 0-1.25% [v/v] n-propanol, 1.6 M (NH₄)₂SO₄ at pH 7.5 at 16-18 °C and appeared within two days. Twin-Strep-tagged FdsBG complex was crystallized over 2-3% [v/v] isopropanol, 1.4 M (NH₄)₂SO₄ at pH 7.5 at 14 °C and appeared within three days.

Crystals were stabilized by successive addition of reservoir solution containing no isopropanol but...
increasing concentrations of glycerol, to a final concentration of 24 % [v/v]. The thus stabilized crystals were mounted and flash-frozen in liquid nitrogen. For NADH-bound FdsBG complex, stabilized Twin-Strep-tagged FdsBG crystals were soaked for an additional 45 min in final stabilization solution containing 10 mM NADH, before mounting and flash-freezing in liquid nitrogen.

**Data collection** – X-ray diffraction data of native FdsBG crystals were collected at the Se-absorption edge (0.97-1.00 Å) on 5.0.1 and 5.0.2 beamlines at the Advanced Light Source (ALS) at Lawrence Berkeley National Laboratory (LBNL) and on 24 ID-C and 24 ID-E beamlines at the Advanced Photon Source (APS) at Argonne National Laboratory (ANL). Anomalous X-ray diffraction data was collected at the Fe absorption peak wavelength of the intrinsic iron-sulfur clusters at 1.7388 Å on 5.0.2 beamline at ALS at LBNL, on 24 ID-C beamline at APS at ANL, and on BL7-1 beamline at the Stanford Synchrotron Radiation Lightsource (SSRL) at the SLAC National Accelerator Laboratory. Collected data were processed with HKL-3000 (37), DIALS (38), XDS (39), and Mosflm (40).

**Structure determination** – The structure was solved to a resolution of ~3.5 Å by single anomalous diffraction-molecular replacement (SAD-MR) using the Crank2 - SAD/MR pipeline (41) as implemented in CCP4 (42). The search model for molecular replacement was generated based on the homology of FdsB and FdsG to Nqo1 and Nqo2 from NADH dehydrogenase of *T. thermophilus* [chain 1 and 2 in the 2FUG pdb file; (11)] using the SWISS-MODEL webserver (43). To improve the completeness of our high-resolution data set, two native data sets of equivalent diffraction limit were combined using the ‘blend’ program as implemented in CCP4. The initial phases generated by SAD-MR were extended and refined to 2.3 Å of our high resolution native FdsBG data set using AutoBuild and Refine as implemented in the PHENIX program suite (44,45).

The solution of the X-ray structure shows two molecules of FdsBG complex in the asymmetric unit which are related by an improper non-crystallographic rotation axis of ~179°. This improper rotation axis is nearly perpendicular (~92°) to the crystallographic two-fold axis (Fig. 2A).

The model of the FdsBG complex was built into the high resolution phased electron density with COOT (46) and refined with the PHENIX Refine subroutine (44). The restraints for the FMN and the Fe₃S₄ cluster were generated with eLBOW as implemented in the PHENIX program suite. Rstraints file for the noncubane Fe₂S₄ cluster was taken from the GeoStd restraints file library (https://sourceforge.net/projects/geomdl/). The distance and angle restraints for the covalent bonds between the iron-sulfur clusters and the cysteine residues of their binding sites in the FdsBG complex were derived from high-resolution structures of well-characterized iron-sulfur complexes. Iterative cycles of building and refining were performed until R-factor values converged to an R<sub>work</sub> of 18.2 % and R<sub>free</sub> of 22.3 % (see Table S1 for detailed data and refinement statistics).

The data set of the NADH-soaked crystal was solved by molecular replacement using the above solved FdsBG structure as a starting model. Iterative cycles of building and refining were performed until R-factor values converged to an R<sub>work</sub> of 15.9 % and R<sub>free</sub> of 19.9 % (see Table S1 for detailed data and refinement statistics).

Structure alignments were performed using secondary structure matching as incorporated in COOT and all molecular structure figures were prepared with PyMOL (Version 1.7.4, Schrödinger).

**Inductively coupled plasma optical emission spectrometry (ICP-OES)**

ICP-OES (Optima 7300DV, Perkin-Elmer at the Environmental Sciences Research Laboratory, UCR) was used to identify the metal ions present in a sample of FdsBG. Approximately 220 µl of 240 µM FdsBG complex was buffer exchanged into 5 mM triethanolamine, pH 7.7 then diluted with nitric acid to 65 % [v/v]. The sample was boiled for 10 min and then diluted to 15 ml for analysis (final nitric acid concentration ~ 2-3 %).

**EPR Spectroscopy**

EPR spectra were recorded using a Bruker EMX spectrometer equipped with a Bruker ER 4119HS high sensitivity X-band cavity and gaussmeter, operated with WinEPR version 4.33 acquisition.
Activity measurements and absorbance spectra were performed using a Hewlett-Packard 8452A diode array spectrophotometer equipped with a temperature-controlled cell holder. Reductive titrations were performed with 20 mM buffered sodium dithionite and 50 μM enzyme in 100 mM K-PO₄, at pH 7.0 at room temperature using a custom fabricated anaerobic cuvette with sidearms (51). The level of flavin saturation was determined as previously described, by treatment of protein with 5 % TCA and quantification of released flavin at 446 nm using ε = 11,100 M⁻¹ cm⁻¹ for acidified FMN (52).

### Rapid Reaction Kinetics

The reaction of oxidized FdsBG complex with NADH was followed using a SX-20 stopped flow spectrophotometer (Applied Photophysics Ltd., Leatherhead, Surrey, UK) equipped with a photodiode array and a photomultiplier tube detection and operated with ProData SX 2.2.5.6 acquisition software. Time courses for the reaction were monitored at 450 nm at 5 °C and fitted to a single exponential equation by nonlinear least squares regression analysis using the software ProData Viewer 4.2.0 (Applied Photophysics Ltd., Leatherhead, Surrey, UK).

Only the first 15 – 120 ms of each time course was analyzed. The observed rate constants, kₒbs, were plotted against substrate concentrations to obtain the limiting rate constant for reduction, kᵣₑᵈ, and the dissociation constant, Kᵣ, using the following equation:

\[
k_{\text{obs}} = \frac{k_{\text{red}} [S]}{(K_{\text{r}} + [S])}
\]

For analysis of the second-order disproportionation reaction (the slow phase of the reaction of the enzyme with NADH), a 60 s trace collected at 450 nm was used to first estimate the endpoint of the reaction. A background value was then subtracted to bring the beginning of the second-order process to zero. The trace was then subtracted from the endpoint value and a Δε = 7200 M⁻¹ cm⁻¹ was estimated empirically assuming all 5 μM of NADH were converted to the neutral semiquinone at the end of the fast phase and that disproportion proceeded to completion by the end of the slow phase. The data was then converted to molar concentrations using the Δε calculated above and time vs. reciprocal concentrations was plotted. The second-order rate constant was
calculated by linear regression analysis of the data in the 2 – 5 s range.

Data availability - All atomic coordinates and structure factors can be found in Research Collaboratory for Structural Bioinformatics database (http://www.rcsb.org) under PDB entries: 6VW8 for the FdsBG complex and 6VW7 for the NADH-bound FdsBG complex. All data is included in the manuscript.

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Author contribution - DN developed the purification protocols for FdsABG holoenzyme and FdsBG complex and performed all rapid kinetic experiments. XY and TKT designed and constructed the expression system for FdsABG holoenzyme and FdsBG complex, TY and SH purified, crystallized, and solved the X-ray structure of the FdsBG complex. GB, RH, and TY conceived and designed all X-ray crystallography experiments. TY and GB analyzed all X-ray data and results. RH and DN conceived and designed all kinetic and spectroscopic experiments. RH and DN analyzed all rapid kinetic and spectroscopic data. All authors participated in the writing and approved the final version of the manuscript.

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Conflict of Interest - The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. EPR spectra of the iron-sulfur clusters of the FdsBG complex. A) Observed iron-sulfur EPR spectrum (black trace) and simulated composite spectrum (red trace) of the dithionite-reduced FdsBG complex, collected at 9 K with modulation amplitude of 8 Gauss and microwave power of 2 µW. The sample was prepared by incubation of 125 µM of FdsBG complex in 100 mM K-PO₄, at pH 7.0 with 2 mM buffered sodium dithionite under anaerobic conditions for 1 h at room temperature prior to freezing. B) The individual component spectra resulting from the simulation of the composite spectrum in panel A: the spectrum corresponding to the previously assigned Fe/S₁ (blue dashed trace; (6)) and an additional Fe/S₅ signal (red dashed trace) resolved only in the FdsBG complex. C) Superposition and graphical alignment of the spectrum presented in panel A (red trace) with the spectrum of dithionite-reduced FdsABG collected at 20 K (blue trace, expanded to match the amplitude of the g₁ feature in the Fe/S₁ component of the FdsBG spectrum; reprint by permission (6)). Dashed lines mark the location of g₁ and g₃ features of the Fe/S₃ component of the FdsABG holoenzyme spectrum.
Figure 2. Arrangement of the two FdsBG complexes of the asymmetric unit and electron density map of representative regions. A) The placement of the two FdsBG complexes of the asymmetric unit within the crystallographic unit cell. The view is down the ~179° rotation axis between the two FdsBG complexes of the asymmetric unit. Crystallographic axes are in blue, with the b-axis being the crystallographic two-fold. The angle between the ~179° rotation axis and the crystallographic two-fold (i.e., b-axis) is ~92°. B) – E) The (2F,o-Fc) electron difference map for different regions of the final model: B) the α-helical region between residues 234B and 244B (superscript B indicates that residues belong to FdsB) contoured at 1.0 sigma and carve radius of 2.0 Å, C) the FMN bound to FdsB contoured at 1.0 sigma and carve radius of 1.5 Å, D) the Fe₄S₄ cluster of FdsG contoured at 1.0 and 6 sigma and carve radius of 2.5 Å, and E) the Fe₄S₄ cluster of FdsB contoured at 1.0 and 6 sigma and carve radius of 2.0 Å. The electron difference map contoured at 1.0 and 6.0 sigma are shown as light-blue and blue meshes, respectively.
Figure 3. Domain structure of FdsB and FdsG and their sequence and structure alignment with NuoE and NuoF. A) Primary structures of FdsB, Nqo1 from *T. thermophilus* and NuoF from *A. aeolicus*. The domains are indicated as boxed regions. The FdsB specific N-terminal thioredoxin-like domain (Txr-like), N-terminal region of Nqo1, Rossmann-like fold, ubiquitin-like domain, and four-helical bundle (4-HB) are shown in brown, light green, white, green, and beige. B) Primary structures of FdsG, Nqo2, and NuoE. The N-terminal four-helical bundle and the thioredoxin-like domain (Txr-like) are shown in brown and light-green. C and D) Structures of FdsB and FdsG subunits with domains color coded according to (A) and (B). E) Superposition of FdsB (beige) and NuoF (light-green) subunits, of FdsG (brown) and NuoE (green) subunits, and of FdsBG (beige and brown) and NuoEF complexes (light-green and green).
Figure 4. Structural comparison of the FdsBG and NuoEF complexes. A and B) The effect of the structural difference in the 183B-190B loop of the Rossmann-like domain on the positioning of the Fe$_2$S$_2$ cluster (A), of the ubiquitin and four-helical bundle domains and of the Fe$_4$S$_4$ cluster (B). The Rossmann-like, ubiquitin, and the four-helical bundle domains of FdsB are displayed in white, green, and brown, and the C-terminal domain of FdsG in gold. The corresponding domains of NuoF and NuoE are shown in gray, light-green, beige, and pale-yellow. C) Difference in the C- and N-terminal domain arrangement of FdsG (brown and green) and NuoE (beige and light-green). D) Hinging of FdsG’s C-terminal domain towards the Rossmann-like domain of FdsB compared to NuoE’s C-terminal domain. E) and F) The overall arrangement of the Fe$_2$S$_2$ cluster, Fe$_4$S$_4$ cluster, and FMN in FdsBG (E) and NuoEF (F).
Figure 5. FMN binding site. The binding site of the flavin ring (A) and of the ribityl-phosphate moiety (B and C) of FMN are displayed. The Fe$_4$S$_4$ cluster of FdsB is rendered in space-fill representation, while FMN, residues of the binding site, and coordinating water molecules are rendered in ball-and-stick representation. FMN, selected residues of FdsB, and water molecules are shown in gold, beige, and red, respectively.
Figure 6. Reductive titration of FdsBG complex at pH 7.0.  

A) Oxidized (red) and sodium dithionite-reduced (blue) spectra.  
B) Change in absorbance as a function of reduction.  

The inset plots the relative absorbance change at 550 nm (y-axis) against relative absorbance change at 450 nm (x-axis) with the diagonal reflecting strict proportionality in the absorbance change between two wavelengths.  

The titration was performed at room temperature in 100 mM K-PO₄, at pH 7.0 under anaerobic conditions.
Figure 7: NADH binding site. A) The unbiased (F_o-F_c)- and (2F_o-F_c)-electron difference maps for FdsBG crystals soaked with NADH. The final models for FMN and the adenosine diphosphate of NADH are displayed in ball-and-stick (gold and cyan). The (F_o-F_c)-electron difference map is contoured at 3.0 sigma with carve radius of 1.8 Å and the (2F_o-F_c)-electron difference map is contoured at 1.0 and 3.0 sigma with carve radius of 1.8 Å are shown in pale green, light blue, and blue meshes, respectively. B) Steric overlap between the D184B-E185B peptide bond with FMN in crystals of FdsBG complex soaked with NADH. The position of the D184B-E185B peptide bond shown was determined for FdsBG complex without FMN bound. C) Comparison of the NADH/NAD\(^+\) positions relative to the FMN of the NADH-bound FdsBG and the NAD\(^+\)-bound NuoEF complex. Shown are the FMN and NAD\(^+\) from the NuoEF complex (pale cyan and light yellow; [6hli.pdb]) and of the NADH of FdsBG complex (cyan). D) The binding site of adenosine diphosphate of NADH. Displayed are FMN, NADH and the adenosine binding site on FdsB in gold, blue, and beige. Residues V295B-A297B and K292B are omitted from this view for clarity.
Figure 8. Single wavelength pre-steady-state kinetics for the reduction of FdsBG with NADH. Plot of $k_{\text{obs}}$ (black circles) versus NADH concentrations. Hyperbolic fits (solid line) yielded a $k_{\text{red}}$ of 680 s$^{-1}$ and $K_d$ of 0.19 mM. Each point is the average of three to five measurements and the error bars are the standard deviation of these measurements. Inset shows a typical trace for the reaction of 9 µM FdsBG complex with 14 µM NADH monitored at 450 nm. All reactions were performed at 5 °C in 100 mM K-PO$_4$, at pH 7.0 under anaerobic conditions.
Figure 9. Rapid reaction kinetics for the reaction of FdsBG with NADH. A) Selected traces for the reaction of 10 µM of FdsBG complex with 5 µM NADH at 5 °C in 100 mM K-PO₄, at pH 7.0 performed under anaerobic conditions and monitored with a photodiode detector. The oxidized spectrum was obtained by diluting FdsBG complex with buffer in the stopped-flow instrument. Inset shows a time course extracted at 450 nm. B) Difference spectra from the data presented in panel (A).
Figure 10. EPR of the neutral flavin semiquinone, FMNH• of FdsBG.  A) Sample was prepared by rapid-freeze quench of a reaction of 40 µM enzyme with 0.8 mM NADH at 0 °C (quenching time ~40 ms). The EPR spectrum was collected at 150 K with modulation amplitude of 8 Gauss and microwave power of 0.4 mW. B) Sample was prepared by mixing 160 µM of FdsBG with 60 µM NADH at room temperature for 10 s prior to freezing. The EPR spectrum was collected at 9 K with modulation amplitude of 8 Gauss and microwave power of 2 µW. All samples were prepared in 100 mM K-PO₄, at pH 7.0 under anaerobic conditions.
Figure 11. Sequence and structure comparisons of FdsBG, NuoEF, and HoxF. A) Comparison of the domain structure of FdsB, FdsG, NuoE, NuoF, and HoxF. B) Sequence alignment of the FdsB, NuoF, and HoxF regions for which an oxidation state-dependent peptide flip has been reported in NuoEF. C) Structure comparison of FdsBG in the presence and absence of NADH. D) Structure of NuoEF by itself (6HL2.pdb) and in oxidized form with bound NAD$^+$ (6HL3.pdb). E) Structure of HoxFUHY in reduced form (5XFA.pdb) and oxidized form (5XF9.pdb). Shown is the mainchain in the region of interest for each protein. The “reduced” and “oxidized” form of each protein is shown in beige and brown, respectively. Also indicated with (“-FMN”) is the absence of FMN in the NADH-bound FdsBG and H$_2$-reduced HoxFUHY structures.
Scheme 1: Proposed electron transfer in FdsBG upon NADH reduction.
Crystallographic and kinetic analyses of the FdsBG subcomplex of the cytosolic formate dehydrogenase FdsABG from Cupriavidus necator
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