Flavin Interaction in NADPH-Sulfite Reductase

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E. coli NADPH-sulfite reductase, depleted of FMN but retaining its FAD, has been prepared
by photoirradiation of native enzyme in 30% — saturated ammonium sulfate. FMN-depleted
enzyme loses its ability to reduce (using NADPH) ferricyanide, cytochrome c, sulfite, or the
enzyme's own heme-like chromophore. However, the FAD remains rapidly reducible by NADPH,
and the FMN-depleted enzyme retains NADPH-acetylpyridine NADP\(^+\) transhydrogenase activity.
Thus, FAD can serve as entry port for NADPH electrons, and FMN is required for further trans-
mission along the enzyme's electron transport chain. These data, plus other studies, have enabled
us to suggest a mechanism for catalysis which involves FAD cycling between the fully-oxidized
and fully-reduced forms while FMN cycles between fully-reduced and semiquinone. This
mechanism, which includes a disproportionation step, permits a “step-down” from the two-
electron donor, NADPH, to a succession of equipotential one-electron transfer steps.

Sulfite reductase from *Escherichia coli* and *Sal-
monella typhimurium* catalyzes the six-electron re-
duction of sulfite to sulfide, using NADPH as the
electron donor \(^1,2\). The enzyme is isolated as a
soluble but high molecular-weight (MW = 670,000)
protein containing an array of prosthetic groups
whose function appears to be the transport of elec-
trons from the donor, NADPH, to a heme-like
chromophore which serves as the sulfite reduction
site \(^1,3\). The enzyme contains, per mole, 4 FMN,
4 FAD, 20 moles of iron (four of which are as-
associated with the heme-like chromophore) and ap-
proximately 14 labile sulfides \(^*\). This complex
system is accommodated on twelve peptide chains,
which appear to be of only two types \(^3,4\).

Four chains (termed “\(\beta\)”) bind all of the iron-
containing groups including the heme-like chromo-
phore. This iron-containing polypeptide, which has
been prepared free of the flavoprotein moiety (both
from *Salmonella* mutants and from urea-dissociated
wild-type *E. coli* enzyme) is capable of reducing
sulfite to sulfide with reduced methyl viologen (but
not NADPH) as electron donor \(^3\). The remaining
eight chains (termed “\(\alpha\)”) bind the FAD and FMN
groups. The \(\alpha\)-octamer flavoprotein, which has been
isolated in pure form from *Salmonella* mutants, can
catalyze a variety of NADPH-dependent reactions
including reduction of cytochrome c, ferricyanide
and other “diaphorase-type” acceptors, as well as
the transfer of electrons from NADPH to 3-acetyl-
pyridine NADP\(^+\) (APyNADP\(^+\)) in a transhydro-
genase-type reaction. The flavoprotein cannot cata-
yze reduction of sulfite by either NADPH or re-
duced methyl viologen.

The presence of multiple flavins, including both
FMN and FAD, raises the possibility that flavin-
flavin interactions may be an integral part of elec-
tron transport in this enzyme. Previous studies with
NADPH-sulfite reductase from entero-bacteria \(^1\) and
yeast \(^5\) have indicated that the FAD and FMN
groups do not serve identical roles.

Although native sulfite reductase binds FMN and
FAD in equal amounts, recent experiments have
shown that FMN binding is considerably weaker
than that of FAD. When sulfite reductase from *E.
coli* is serially diluted in the range \(10^{-8}\) to \(10^{-9}\) M
total flavin, the fluorescence intensity per mole (ex-
citation at 450 nm and emission at 535 nm) in-
creases markedly. Ultrafiltration of dilute enzyme
solutions yields FMN but no FAD in the filtrate.
The dissociation constant of FMN obtained from
these data was \(1.0 \times 10^{-8}\) M at 25° in 0.05 M
potassium phosphate buffer, pH 7.7, containing
\(1 \times 10^{-4}\) M EDTA (“standard buffer”). All four of

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\(^*\) Ref. 3 suggests about 16 iron atoms, but more recent
unpublished results, obtained in collaboration with Dr.
MATTHEW J. MURPHY, make the number twenty (with 4
hemes) more probable.

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the FMN moieties appear to be equivalently dissociable from the native enzyme. Accompanying this dissociation of FMN is the development of FMN-stimulability of NADPH-cytochrome c activity upon dilution of enzyme. Thus, the loss of FMN appears to be catalytically significant.

We have taken advantage of the relatively high dissociability of FMN to prepare large quantities of FMN-depleted sulfite reductase, which could be used to assess the function of the two types of flavin in electron transfer. In a modification of the procedure of Baggott and Langdon, the enzyme, placed in 30% saturated ammonium sulfate in standard buffer, was irradiated with a bank of fluorescent lights to destroy dissociated FMN by photolysis. Analysis of irradiated enzyme showed that FMN was indeed destroyed by this procedure, with a half-time of about 2 hours in an apparent first-order reaction at 4°C with an initial enzyme concentration of 2 × 10^{-8} M in flavin. The FAD content was affected far less by this treatment. Enzyme irradiated for approximately 10 hours consistently retained at least 85% of its original FAD, but less than 5% of the initial FMN.

The FMN-depleted enzyme loses the ability to catalyze reduction of cytochrome c, ferricyanide, or sulfite by NADPH. The rate of loss of these activities closely parallels the loss of FMN. Furthermore, NADPH can no longer reduce the heme-like chromophore of sulfite reductase. Addition of FMN to depleted enzyme restored 50 to 75% of the NADPH-cytochrome c reductase activity of untreated enzyme, with an apparent K_m for FMN of 1.1 × 10^{-8} M. The irradiated enzyme quenched the fluorescence of added free FMN with an apparent dissociation constant similar to the K_m for stimulation of cytochrome c reduction, and to the previously-cited dissociation constant obtained from dilution of native enzyme. Addition of excess FMN to irradiated enzyme also restored the ability of the heme-like chromophore to be reduced by NADPH. The latter phenomenon has not yet been studied in detail.

Two pyridine nucleotide-linked activities remain viable in FMN-depleted enzyme: The transhydrogenase activity remains, and the FAD remains rapidly reducible by NADPH. Electron transfer between NADPH and APyNADP⁺ was virtually unaffected by the removal of over 95% of the enzyme-FMN; furthermore, in contrast to the marked FMN stimulation of NADPH-dependent reductase activities in the depleted enzyme, the transhydrogenase activity was unaffected by restoration of FMN.

The residual FAD of irradiated enzyme was reducible (to FADH₂) upon addition of excess NADPH. The rate of this reduction, when examined in the stopped-flow apparatus, was at least as great as in native enzyme. Thus, FMN is not required for the introduction of NADPH electrons into the enzyme. If transhydrogenase activity is an index of the initial event in the process of electron flow, then the retention of this activity in FMN-depleted enzyme strengthens the supposition that FAD is the flavin which first receives electrons from NADPH. While the FMN is not required for the injection of NADPH electrons into the enzyme, its presence appears to be essential for the further transfer of these electrons from FAD to the enzyme-heme (and ultimately to sulfite), as well as to artificial electron acceptors such as cytochrome c and ferricyanide.

The electron acceptors for whose reduction FMN has thus far been shown to be required, can all be reduced via one-electron transfers, and thus it is tempting to speculate that the FMN-FAD interaction is part of a “step-down” process from the two-electron donor, NADPH, to one-electron acceptors. Interaction between two flavin moieties, each undergoing one-electron alterations, has been proposed for the “step-down” reaction in microsomal NADPH-cytochrome c reductase, and Hemmrich et al. have proposed such interactions as general mechanisms for these types of processes.

Previous studies with the flavoprotein moiety of sulfite reductase, obtained from Salmonella mutants, suggested the participation of more than one type of flavin redox cycle in the transfer of electrons from NADPH to ferricyanide. These studies indicated that, following enzyme turnover with an excess of both NADPH and ferricyanide, one electron per flavin pair was retained in the enzyme (as flavin semiquinone) after exhaustion of the NADPH. This flavin semiquinone was then oxidized by ferricyanide in a reaction far too slow to be of catalytic significance. These results have now been confirmed and extended with the wild-type E. coli enzyme; the state of the enzyme flavin following turnover (3/4 oxidized flavin, 1/4 flavin semiquinone) remains constant over a range from 1 to 100 NADPH per enzyme-flavin so long as the ferricyanide remains in a stoichiometric excess.
When cytochrome c serves as electron acceptor, similar results are obtained; these experiments have the further advantage that the large extinction coefficient of reduced cytochrome c can be utilized in quantitation instead of the small extinction coefficient of flavin semiquinone. Comparison of the number of reducing equivalents presented as NADPH to the equivalents appearing as reduced cytochrome c, permits calculation of the number of electrons remaining with the enzyme after turnover.

When sulfite reductase, $1 \times 10^{-5}$ M in flavin, was reacted with NADPH and excess cytochrome c in the stopped flow apparatus, a stoichiometry of $1.5 \pm 0.1$ moles of reduced cytochrome c per NADPH was obtained when equimolar quantities of pyridine nucleotide and enzyme flavin were used. With low NADPH to flavin ratios, the reduced cytochrome c to NADPH stoichiometry approached one (with NADPH/flavin = 0.25, cytochrome c/NADPH = $1.2 \pm 0.1$, uncorrected for the absorbance increase at 550 nm from formation of flavin semiquinone). With high NADPH/flavin ratios, the stoichiometry approached 2 (with NADPH/flavin = 6, cytochrome c/NADPH = $1.8 \pm 0.1$).

These observations support the mechanism shown below, where $F_A = FAD$ and $F_B = FMN$, and $A = $ one-electron acceptor.

\[
\begin{align*}
1 & \rightarrow F_A F_B H^+ A H^+ NADPH \rightarrow F_A H_2 F_B H^+ A H^+ (1) \\
2 & \rightarrow F_A H_2 F_B H^+ A H^+ (7) \\
3 & \rightarrow F_A F_B H^+ (3) \\
4 & \rightarrow F_A H^+ F_B H^+ NADPH (4) \\
5 & \rightarrow F_A F_B H^+ A H^+ (5) \\
6 & \rightarrow F_A H_2 F_B H^+ (6)
\end{align*}
\]

In this scheme, steps (7) and (8) represent alternate disproportionation reactions; NADPH always reacts with a fully-oxidized FAD [steps (1) and (4)], and electron acceptor always reacts with fully reduced FMN [steps (3) and (6)]. In effect, then, the FAD cycles between the fully reduced and fully-oxidized states (probably via a semiquinone), while the FMN cycles only between fully reduced and semiquinone forms during catalysis. As suggested by Hemmerich (discussion of ref. 3), this type of mechanism provides a means whereby the sites of the multielectron reduction of sulfite to sulfide can obtain single electrons of constant potential from NADPH via the flavin groups.

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