Interaction of 2-n-Heptyl-4-Hydroxyquinoline-N-Oxide with Dimethyl Sulfoxide Reductase of Escherichia coli*

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We have studied the interaction of the menaquinol analog 2-n-heptyl-4-hydroxyquinoline-N-oxide (HOQNO) with dimethyl sulfoxide reductase (DmsABC) and the effect of a mutation in the DmsC subunit (DmsABC<sup>C165R</sup>) using fluorescence titration and stopped-flow methods. The titration data show that the HOQNO fluorescence is quenched when HOQNO binds to DmsABC. The binding stoichiometry is determined to be about 1:1. The mutant DmsABC<sup>C165R</sup> blocks HOQNO binding to the protein. It is therefore proposed that there is one high-affinity HOQNO binding site per DmsABC molecule located in the DmsC subunit. Stopped-flow kinetic studies show that the interaction can be described by a two-step equilibrium model, a fast bimolecular step followed by a slow unimolecular step. The quenching of HOQNO fluorescence occurs in the bimolecular step. The rates for the forward and reverse reaction of the first equilibrium are determined to be \( k_1 = (3.9 \pm 0.3) \times 10^6 \text{ M}^{-1} \text{s}^{-1} \) and \( k_2 = 0.10 \pm 0.01 \text{ s}^{-1} \), respectively. The dissociation constant for the first equilibrium, \( K_d = k_2/k_1 \), is calculated to be about 280 nm. The upper limit of the overall dissociation constant is estimated to be 6 nm.

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The abbreviations used are: DmsABC, dimethyl sulfoxide reductase (DmsABC<sup>C165R</sup>); HOQNO, 2-n-heptyl-4-hydroxyquinoline-N-oxide; MQH<sub>2</sub>, menaquinol; EPR, electron paramagnetic resonance; MOPS, 3-(N-morpholino)propanesulfonic acid.

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by a modified Lowry assay in the presence of 1% SDS using a Bio-Rad
serum albumin protein standard (17). The concentration of DmsABC
was calculated from the total concentration of [4Fe-4S] clusters mea-
sured by the EPR spin quantitation and by assuming that there are four
[4Fe-4S] clusters per DmsABC molecule (18, 19).

Quenching of HOQNO Fluorescence by Binding to DmsABC—HOQ-
NO was obtained from Sigma. The concentration of HOQNO was
determined spectrophotometrically after diluting the ethanolic stock
solution in 1 mM sodium hydroxide using an extinction coefficient of
9450 M⁻¹ cm⁻¹ at 346 nm (20). Fluorescence measurements were car-
ried out using a Perkin-Elmer LS-50B luminescence spectrometer. The
fluorescence emission spectrum of HOQNO in 100 mM MOPS and 5 mM
EDTA (pH 7.0) exhibits a maximum at 479 nm with the excitation at
341 nm (data not shown). In fluorescence titration experiments, ali-
quots of a 50 μM HOQNO stock solution were added to the cuvette
containing the protein in 100 mM MOPS and 5 mM EDTA (pH 7.0), and
fluorescence emissions were measured at 479 nm (with excitation at
341 nm). The background fluorescence of the protein sample in the
absence of HOQNO was subtracted from the fluorescence of the sample
in the presence of HOQNO.

Stopped-flow Experiment and Data Analysis—The stopped-flow ex-
periments were performed using a Sequential Bio SX-17MV stopped-
flow spectrofluorimeter (Applied Photophysics Ltd., Leatherhead, UK).
In a typical experiment, DmsABC or DmsABC(H65R) (1 or 2 μM) in 100
mM MOPS and 5 mM EDTA (pH 7.0) was mixed with an equal volume
of various concentrations of HOQNO (1–14 μM) in the same buffer.
Temperature was maintained at 25 °C. The fluorescence of HOQNO
was excited at 341 nm, and the emissions above 400 nm were recorded
through a cut off filter SGG-400-1.00 (CVI Laser Co., Albuquerque,
NM). For each concentration of HOQNO, at least three runs were
performed and 1000 or 2000 data points were collected. After averaging,
data were fitted to an appropriate equation using the software supplied
by Applied Photophysics. Under our experimental conditions, quench-
ing of HOQNO fluorescence by binding to DmsABC was biphasic, and
the observed fluorescence, F, was best fitted to a double exponential
equation,

\[ F = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + b \]  

(Eq. 1)

where \( A_1 \) and \( A_2 \) are the amplitudes of the fast and slow phase, and \( k_1 \)
and \( k_2 \) are the observed rates for the fast and slow phase, respectively,
\( t \) is time and \( b \) is an off set value of the stopped-flow instrument.

The interaction of HOQNO with DmsABC can be described by the
following model (21–23) (see “Discussion”),

\[ k_1 \]

\[ \frac{E}{L} \rightleftharpoons EL \rightleftharpoons EL^* \]  

(Eq. 2)

where \( E \) represents DmsABC; \( L \) the ligand (HOQNO); \( EL \), the initial
complex of DmsABC with HOQNO before the isomerization takes place;
\( EL^* \), the final complex product; \( k_1 \) and \( k_2 \) are the rate constants for the
forward reactions in \( s^{-1} \) and \( s^{-1} \), respectively, and \( k_3 \) and \( k_4 \) are the
rate constants for the reverse reactions in \( s^{-1} \). In the case where the
bimolecular process is much faster than the unimolecular process
and the initial concentration of \( E \) is much higher than the concentration
of \( L \), the observed first-order rates for the fast phase (\( k_f \)) and the slow
phase (\( k_s \)) are given by Equations 3 and 4, respectively,

\[ k_f = k_2 + k[L] \]  

(Eq. 3)

\[ k_s = k_4 + k(1 + K_d[L]) \]  

(Eq. 4)

where \( K_d \) is the dissociation constant of the first equilibrium in
Equation 2.

Under our experimental conditions, the plot of HOQNO fluorescence
against the concentration of HOQNO was linear up to 7 μM of HOQNO
(after mixing) (data not shown). This was, therefore, the highest con-
centration of HOQNO used in this work. Toward the low end of the
HOQNO concentrations used herein, the condition that initial concen-
tration of \( L \) is much higher than the concentration of \( E \) (0.5 μM after
mixing) does not hold. However, the observed first-order rates for the
fast phase, \( k_f \), can still be analyzed using Equation 3 as demonstrated by
Halford (21).

From the fit of the observed \( k_f \) data to Equation 3, \( k_1 \) and \( k_2 \) can be
obtained. Under our experimental conditions, deviations of the ob-
served \( k_f \) were too large to be fitted to Equation 4 to yield reliable \( k_f \)
and \( k_s \). To evaluate the values of \( k_f \) and \( k_s \), another approach using the Glint
program (Applied Photophysics) was employed. This program enables
one to globally analyze a complete data set measured at all wavelengths
according to a proposed reaction scheme and to obtain the reaction
parameters from the best fit of the calculated data to the experimental
data. Kinetic data irrelevant of wavelength (the case of this work) or
measured at a single wavelength can also be analyzed using this pro-
gram. In this work, using the reaction scheme (Equation 2), and the \( k_1 \)
and \( k_2 \) determined from Equation 3, kinetic traces measured at various
HOQNO concentrations were analyzed using the Glint program, and
the values of \( k_f \) and \( k_s \) were evaluated from the good fits of the calcu-
lated kinetic traces to the observed traces.

RESULTS

Stoichiometry of HOQNO Binding to DmsABC—The binding of
HOQNO to DmsABC was examined by measuring the HOQ-
NO fluorescence emission (at 479 nm with excitation at 341
nm) in the absence and in the presence of DmsABC. In the
absence of DmsABC, the plot of fluorescence measured by
titrating the buffer (100 mM MOPS and 5 mM EDTA, pH 7.0)
against the concentration of HOQNO gave a straight line with
a positive slope as shown in Fig. 1A. This fluorescence was
attributed to free HOQNO molecules (not bound to other com-
pounds). In the presence of DmsABC, however, the initial por-
tion of each titration curve was flat (Fig. 1A), indicating that
there was no increase of fluorescence intensity upon the addi-
tion of a certain concentration of HOQNO to the protein. In
other words, there was no free HOQNO present in the system
to emit fluorescence. It was clear that the fluorescence of HOQ-
NO was quenched in the presence of DmsABC and this
quenching was because of HOQNO binding to DmsABC. Fur-
ther addition of HOQNO to the protein caused a sudden in-
crease of fluorescence intensity, suggesting that free HOQNO
became available when the amount of HOQNO added to the
protein was higher than a certain concentration at a given
concentration of DmsABC.

It was not possible to construct a Scatchard plot from the
titration data in Fig. 1A because of the sharp transition of
the titration curves. To obtain the stoichiometry of HOQNO bind-
ing to DmsABC, four concentrations of DmsABC (0.25, 0.5,
0.75, and 1.0 μM) were titrated with HOQNO in 100 mM MOPS
and 5 mM EDTA at pH 7.0 (Fig. 1A). The extrapolations of the
linear portions of the titration curves to zero fluorescence yield
four intercepts on the x-axis of the plot. These intercepts rep-
resent the concentrations of HOQNO bound to DmsABC at the
given concentrations of DmsABC. Therefore, from the plot of the
intercepts against the concentration of DmsABC, Fig. 1B, the stoichiometry of HOQNO binding to DmsABC can be
determined to be approximately 1:1.

Effect of DmsABC(H65R) Mutation on HOQNO Binding to the
Protein—It has been suggested in a recent EPR study (5) that
residue His-65 in the DmsC subunit may be involved in MQH₂
binding and a His→Arg mutation, DmsABC(H65R), may block
binding the MQH₂ analog HOQNO to the protein. To verify
this, titrations of 0.4 μM wild-type DmsABC and mutant
DmsABC(H65R) with HOQNO in 100 mM MOPS and 5 mM EDTA
(pH 7.0) were carried out and the data obtained were compared
as shown in Fig. 2. For the wild-type protein, the initial portion
of the titration curve was flat indicating quenching of HOQNO
fluorescence because of HOQNO binding to the protein. Fur-
ther increase of HOQNO concentration caused a sharp increase
of fluorescence intensity. For the mutant DmsABC(H65R), how-
ever, the titration data gave a straight line with a positive slope,
indicating that there was no quenching of HOQNO fluorescence.
In other words, in the case of titration of DmsABC(H65R), the
HOQNO added to the protein was in the free form and not bound
to the protein. Fig. 2, thus, demonstrates that the mutation of
DmsABC(H65R) blocks HOQNO binding to the protein.

Stopped-flow Studies of Interaction of HOQNO with DmsABC—To investigate the kinetics and mechanism of the
interaction of HOQNO with DmsABC, the stopped-flow fast kinetic technique was employed. Fig. 3 shows a plot of the observed fluorescence intensities against the concentration of HOQNO obtained by mixing various concentrations of HOQNO in buffer of 100 mM MOPS and 5 mM EDTA (pH 7.0) with the same buffer or with 2 mM wild-type DmsABC (in the buffer).

After mixing the protein with HOQNO, a decrease of fluorescence intensity with time was observed as shown in the inset of Fig. 3 indicating quenching of HOQNO fluorescence. The magnitude of the initial fluorescence intensity measured immediately after mixing HOQNO with DmsABC was very similar to the magnitude of the fluorescence intensity measured in the absence of the protein, indicating that no detectable reaction occurred during the dead time of the stopped-flow instrument. The same results were also obtained by mixing 1 mM of DmsABC with various concentrations of HOQNO in 100 mM MOPS and 5 mM EDTA (pH 7.0) under the conditions in Figs. 5B and 6 (data not shown). On the other hand, for the final fluorescence intensity measured 50 s after mixing, the initial portion of the curve was flat and the intercept on the x-axis (about 1 µM) obtained by extrapolating the linear portion of the curve to zero fluorescence was in good agreement with the 1:1 binding stoichiometry obtained under the steady-state conditions (Figs. 1 and 2). Fig. 3 demonstrates that the fluorescence quenching observed after mixing HOQNO with DmsABC is because of HOQNO binding to the protein.
Interaction of HOQNO with DmsABC

The DmsABC<sup>H65R</sup> mutant was used as a non-HOQNO binding control for this work. Fig. 4 shows the plot of the fluorescence intensities against the concentration of HOQNO obtained by mixing 2 μM mutant DmsABC<sup>H65R</sup> with various concentrations of HOQNO in 100 mM MOPS and 5 mM EDTA (pH 7.0). In contrast to Fig. 3, the plot of the initial fluorescence intensity (measured immediately after mixing) and the final fluorescence intensity (measured 50 s after mixing) against the concentration of HOQNO gave two straight lines that not only had the same slope but also overlapped each other within experimental error. Clearly, there was no significant quenching of HOQNO fluorescence in this case. Fig. 4 indicates that the mutation of DmsABC<sup>H65R</sup> blocks HOQNO binding to the protein, which agrees with the steady-state titration data (Fig. 2). Furthermore, this result serves as a good control for the stopped-flow experiment, confirming that the nonlinear feature of the curve for the final fluorescence intensity in Fig. 3 is not because of an artifact. As shown in Fig. 3, binding HOQNO to DmsABC causes a quenching of HOQNO fluorescence, and this quenching process can be followed using the stopped-flow method. To further investigate the kinetics of HOQNO binding to DmsABC, 1 μM DmsABC was rapidly mixed with various concentrations of HOQNO using the stopped-flow method. Fig. 5A shows a typical trace of fluorescence quenching observed after mixing 1 μM of DmsABC with 2 μM HOQNO in 100 mM MOPS and 5 mM EDTA at pH 7.0 (25 °C) and the residuals for fitting these data to Equation 1. The fluorescence quenching process was completed about 50 s after mixing. The observed quenching trace had two phases, a fast phase followed by a slow phase, and it was best fitted with the double exponential equation, Equation 1. This was also true at other HOQNO concentrations used in this work (Fig. 5B). From the fit, the observed first-order rates and the amplitudes for the fast and slow phase at the given concentration of HOQNO, k<sub>f</sub>, k<sub>s</sub>, A<sub>f</sub>, and A<sub>s</sub>, can be determined. The first-order rate for the fast phase (k<sub>f</sub>) observed by mixing 1 μM DmsABC with various concentrations of HOQNO in 100 mM MOPS and 5 mM EDTA at pH 7.0 (25 °C) was plotted against the concentration of HOQNO and the data obtained were fitted to Equation 3 as shown in Fig. 5B, from which the rate constants for the forward and reverse reactions of the first equilibrium in Equation 2 were determined to be k<sub>f</sub> = (3.9 ± 0.3) × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup> and k<sub>r</sub> = 0.10 ± 0.01 s<sup>−1</sup>. Thus the dissociation constant for the first equilibrium, K<sub>A1</sub> = k<sub>r</sub>/k<sub>f</sub>, was calculated to be about 260 nM.

The rate constants for the forward and reverse reactions of the second equilibrium in Equation 2, k<sub>3</sub> and k<sub>4</sub>, were evaluated with the Glint program (Applied Photophysics). Using the reaction scheme described in Equation 2, and the k<sub>1</sub> = (3.9 × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup>) and k<sub>2</sub> = 0.10 s<sup>−1</sup> determined from the fit as shown in Fig. 5B, k<sub>3</sub> = 0.40 ± 0.04 s<sup>−1</sup> and k<sub>4</sub> = 0.01 s<sup>−1</sup> were obtained from the good fits of the calculated kinetic traces to the observed traces (data not shown). Therefore, the association constant of the second equilibrium, K<sub>A2</sub> = k<sub>3</sub>/k<sub>4</sub> = 40, was
bound complex, $EL^*$, by a slow unimolecular process (isomerization). The isomerization may take place through several possible pathways, such as rearrangement of $EL$ without any conformational change in $E$ or $L$, a conformational change in either $E$ or $L$, or conformational changes in both $E$ and $L$ (22).

It is expected that stopped-flow kinetic studies can provide some useful information about the bimolecular association of HOQNO with DmsABC and the subsequent isomerization process. The effect of HOQNO concentration on the amplitudes of the fast and slow phase may be interpreted as follows. When the concentration of HOQNO is lower than or comparable with the dissociation constant of the first equilibrium in Equation 2, $K_{d1}$, but higher than the overall dissociation constant, only a small amount of protein is associated with the ligand (HOQNO) to form the complex $EL$ by the fast bimolecular process, which is observed as a fast phase of quenching. The subsequent slow isomerization process of converting $EL$ to $EL^*$ would shift the first equilibrium in Equation 2 to the right-hand side slowly. This slow shift of the first equilibrium is observed as a subsequent slow phase of quenching. Alternatively, when the concentration of HOQNO is increased to higher than $K_{d1}$, more and more DmsABC molecules are associated with HOQNO to give $EL$ through the fast bimolecular process, thus, less and less free DmsABC molecules are available for the subsequent shift of the first equilibrium to the right-hand side caused by the slow isomerization process. Therefore, the amplitude of the observed fast phase of quenching increases with increase of HOQNO concentration until becoming saturated, whereas the observed slow phase of quenching decreases with increase of HOQNO concentration, and this would finally result in that the amplitude of the slow phase would be negligible compared with the amplitude of the fast phase. These results suggest that the quenching of HOQNO fluorescence occurs in the bimolecular step of the association of $E$ with $L$ rather than in the unimolecular step of forming $EL^*$ (Equation 2).

Primary dehydrogenases and terminal reductases have been the subject of a large number of steady-state kinetic investigations (see Refs. 24–31 as examples), but rapid reaction methodologies have been limited because of the lack of available probes. We have now shown that fluorescent HOQNO can be used to monitor the quinol binding reaction. Although the steady-state methodology is useful for stoichiometry, the affinity is so tight that Scatchard plots are not useful. The stopped-flow method allows determination of rates and dissociation constants. Although the three-dimensional structures of several quinone-binding proteins have been determined (32, 33), it is not yet possible to define a quinone binding motif. This technique will prove useful in examining site-directed mutations in the quinol binding region or in conformationally coupled changes that alter quinone binding.

This work and a recent study on the interaction of HOQNO with fumarate reductase using EPR and steady-state fluorescence spectroscopic methods (34) demonstrate the utility of the characteristics of HOQNO fluorescence and its structural analogy to MQH$_2$ for studying interactions of MQH$_2$ with terminal reductases in respiratory chains. Studies on the interactions of HOQNO with *E. coli* nitrate reductase$^2$ and with fumarate reductase$^3$ are in progress.

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$^3$ Z. Zhao, R. A. Rothery, and J. H. Weiner, unpublished results.
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