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Subunit functional studies of NAD(P)H:quinone oxidoreductase with a heterodimer approach

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ABSTRACT **NAD(P)H:quinone oxidoreductase (NQOR; EC 1.6.99.2) is a homodimeric enzyme which catalyzes the reduction of quinones, azo dyes, and other electron acceptors by NADPH or NADH. To pursue subunit functional studies, we expressed a wild-type/mutant heterodimer of NQOR in *Escherichia coli*. The wild-type subunit of the heterodimer was tagged with polyhistidine and the other subunit contained a His-194 → Ala mutation (H194A), a change known to dramatically increase the K_m for NADPH. This approach enabled us to efficiently purify the heterodimer (H194A/HNQOR) from the homodimers by stepwise elution with imidazole from a nickel nitrilotriacetate column under nondenaturing conditions. The composition of the purified heterodimer was confirmed by SDS and nondenaturing polyacrylamide gel electrophoresis and immunoblot analysis. The enzyme kinetics of the purified heterodimer were studied with two two-electron acceptors, 2,6-dichloroindophenol and menadione, and a four-electron acceptor, methyl red, as the substrates. With two-electron acceptors, the $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of the heterodimer H194A/HNQOR were virtually identical to those of the wild-type homodimer, but the $k_{cat(NADPH)}$ and $k_{cat(NADH)}$ values were only about 50% those of the wild-type homodimer. With the four-electron acceptor, the K_m and k_{cat} values of H194A/HNQOR for NADPH and NADH were similar to those of the low-efficiency mutant homodimer. These results suggest that the subunits of NQOR function independently with two-electron acceptors, but dependently with a four-electron acceptor. This heterodimer approach may have general applications for studying the functional and structural relationships of subunits in dimeric or oligomeric proteins.**

NAD(P)H:quinone oxidoreductase (NQOR, EC 1.6.99.2), also known as DT-diaphorase, is a flavoprotein widely distributed in animal tissues (1). NQOR is a homodimeric enzyme with two FAD molecules as coenzymes, one for each subunit (2). The enzyme can utilize either NADPH or NADH as an electron donor with similar efficiencies and transfers two or four electrons to quinones, azo dyes, or other electron acceptors (2, 3). The catalysis involves the following sequential steps: (*i*) binding of NAD(P)H to the enzyme and subsequent electron transfer from NAD(P)H to FAD, (*ii*) dissociation of NAD(P) from the reduced enzyme, (*iii*) binding of substrate to the reduced enzyme and subsequent electron transfer from $FADH_2$ to the substrate, and (*iv*) dissociation of the reduced substrate, which is the rate-limiting step (4). The primary structures of rat and human liver NQOR have been deduced from their cDNA sequences, and the sequence of rat liver NQOR was confirmed by protein sequencing (5–8). The calculated subunit molecular mass of the rat enzyme is 30,784 Da. Studies on structure–function relationships have been carried out by chemical modification (8) and site-directed mutagenesis (10–12). The functional relationship of the two subunits, however, is not clear. Our previous results from site-directed mutagenesis studies suggested that a glycine-rich region and His-194 are important in conferring the NAD(P)H specificity of NQOR (ref. 11 and unpublished results). Due to the large increase in $K_{m(NADPH)}$ and decrease in $k_{cat(NADPH)}$ of the enzyme with the His-194 → Ala (H194A) mutation, this mutant was selected for the present subunit functional studies by constructing a heterodimer with the wild-type NQOR.

Studies on the active sites of the homodimer proteins by construction of hybrids of wild-type and site-directed mutant proteins have been reported (13–15). Wente and Schachman (13) used an approach which entails the formation of such hybrids *in vitro*, but this approach is only applicable to proteins which can sustain the dissociation and reassociation conditions. Larimer *et al.* (14) reported the production of a hybrid heterodimer of wild type and a site-directed mutant by plasmid cotransformation. Nevertheless, the study was limited by the fact that the three species of dimeric proteins (wild-type homodimer, mutant homodimer, heterodimer) could not be separated from one another.

In the present study, we have developed an approach to express a heterodimer (H194A/HNQOR) containing a wild-type subunit with a polyhistidine tag at the amino terminus of NQOR and a H194A mutant subunit by constructing two cDNAs under one promoter in *Escherichia coli*. This approach enabled us to separate heterodimer from the two homodimers by stepwise elution with imidazole from a nickel nitrilotriacetate (Ni-NTA) column under nondenaturing conditions. The functions of the heterodimer subunits were studied by characterizing the kinetics of the reduction of 2,6-dichloroindophenol (DCIP), menadione, and methyl red.

MATERIALS AND METHODS

Materials. Ni-NTA agarose was purchased from Qiagen (Chatsworth, CA). The restriction enzymes and DNA ligase were from GIBCO/BRL. Calf intestinal phosphatase was obtained from New England Biolabs. The PCR reagent kit was supplied by Perkin–Elmer/Cetus. Centriprep concentrators were procured from Amicon.

Plasmid Construction. The construction and expression of wild-type NQOR and H194A mutant were described previously (10, 12). For the construction and expression of the His_6-tagged NQOR, the coding sequence of the His_6 tag with a start codon was added to the 5′ end of the wild-type cDNA by PCR. The sequences of the two primers used for PCR to generate wild-type cDNA with His_6 sequence were as follows: primer A, 5′-GGTCAAGCTTTCACACAGGAAACAGAA-ATGCATCACCATCACCATCACGCGGTGAGAA-GAGCCCTGATT-3′; primer B, 5′-GTTTTGGCGGATGAG-AGAAGA-3′. Primer A is composed of three parts: the 5′

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Abbreviations: NQOR, NAD(P)H:quinone oxidoreductase; DCIP, 2,6-dichloroindophenol; Ni-NTA, nickel nitrilotriacetate.
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portion contains a *Hin*dIII restriction site (underlined) and a ribosome binding site (27 nt); the middle portion contains a start codon and the His_6 coding sequence (21 nt); and the 3′ portion contains a partial NQOR cDNA coding sequence. The sequence of primer B is complementary to that of the coding strand of vector downstream of the stop codon. The cDNA fragment with His_6 sequence was thus engineered to contain two *Hin*dIII sites, one before the ribosome binding site and the other after the stop codon of cDNA. For the construction and expression of the heterodimer containing a site-directed mutant subunit and a His_6-tagged wild-type subunit (H194A/HNQOR), the mutant plasmid (bearing H194A cDNA) was linearized by *Hin*dIII restriction enzyme and then dephosphorylated by calf alkaline phosphatase. The NQOR cDNA with a His_6 sequence generated by PCR was cleaved by *Hin*dIII and ligated to the dephosphorylated vector bearing H194A cDNA. This construct would transcribe one continuous mRNA containing the coding sequences for both His_6-tagged NQOR and H194A mutant. The two proteins would be translated separately as two independent subunits within the same host cell.

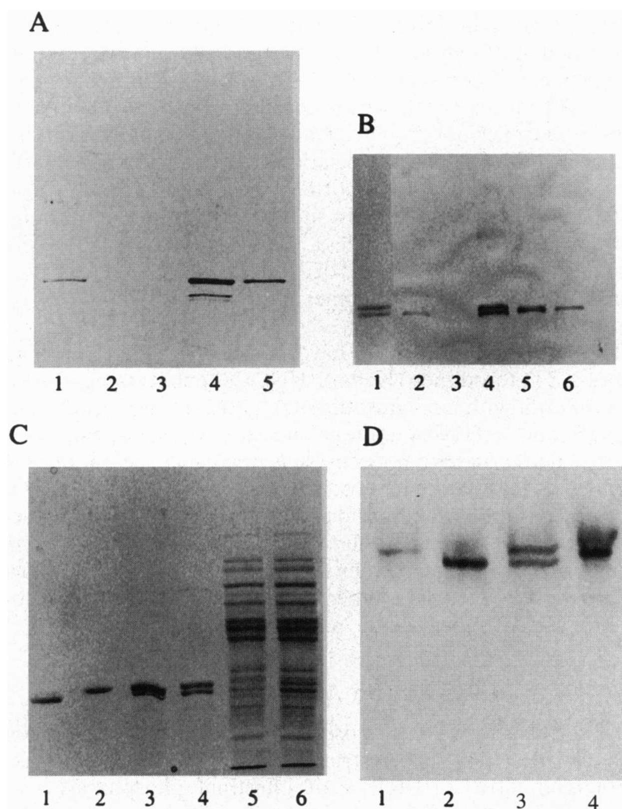

FIG. 1. Electrophoretic analysis of proteins. (*A*) Immunoblot analysis of cell-free extract. Lanes: 1, purified NQOR as a standard; 2, blank; 3, cytosol of expression system with vector plasmid pKK2.7 as a negative control; 4, the H194A/HNQOR expression system; 5, cytosol of the original expression system of NQOR. Each lane contained 0.2 μg of protein. The protein of the lower band in lane 4 is not identified and it may be a degraded product of NQOR or a translation from the internal ATG codon. (*B*) Immunoblot analysis of fractions during purification of the H194A/HNQOR heterodimer. Lanes: 1, mixture of NQOR and HNQOR/HNQOR; 2, flowthrough; 3, wash; 4, fraction eluted in 30 mM imidazole; 5, fraction eluted in 40 mM imidazole; 6, fraction eluted in 50 mM imidazole. (*C*) SDS/polyacrylamide gel electrophoresis and Coomassie blue staining. Lanes: 1, purified NQOR; 2, purified HNQOR/HNQOR; 3, mixture of NQOR and HNQOR/HNQOR; 4, purified H194A/HNQOR; 5 flowthrough; 6, cytosol. (*D*) Nondenaturing polyacrylamide gel electrophoresis of purified dimeric proteins and Coomassie blue staining. Lanes: 1, HNQOR/HNQOR homodimer; 2, NQOR homodimer; 3, mixture of the two homodimers; 4, H194A/HNQOR heterodimer.

The *E. coli* strain AB1899 was transformed with the plasmid DNA by a $CaCl_2$ procedure.

Protein Purification. The cell pellets from 1 liter of overnight culture were suspended in 40 ml of sonication buffer (50 mM sodium phosphate, pH 8.0/300 mM NaCl) and disrupted by sonication. After centrifugation at 12,000 × *g* for 25 min, the supernatant was applied at a flow rate of 1 ml/min onto a 1-ml Ni-NTA column preequilibrated with 50 ml of the sonication buffer. The column was extensively washed at a flow rate of 0.5 ml/min with the sonication buffer and wash buffer (50 mM sodium phosphate, pH 6.0/300 mM NaCl) until the eluent was void of proteins as determined by the Bradford method (16). A single step elution with 0.1 M imidazole yielded a bright yellow fraction (2–3 ml) containing HNQOR/HNQOR. Purification of the heterodimer, H194A/HNQOR, used stepwise elution with 20, 30, 40, and 50 mM imidazole in 10 ml of wash buffer. The resulting four samples were collected and dialyzed overnight against 2 liters of 50 mM sodium phosphate buffer, pH 7.5/0.5 mM EDTA. The samples were then concentrated in a Centriprep concentrator with a molecular size cutoff of 10 kDa.

Enzyme Activity Assays. The DCIP and menadione reductase activities were assayed as described (17). The assay mixture contained 10 ng of purified enzyme and 0.04 mM DCIP, or 0.01 mM menadione and 0.05 mM cytochrome *c*, in a final volume of 1 ml. For DCIP reduction, the activity was determined by following the decrease in A_{600} ($\varepsilon = 2.1 \times 10^4$ $M^{-1} \cdot cm^{-1}$). For menadione reduction, the activity was determined by following the increase in A_{550} ($\varepsilon = 1.85 \times 10^4$ $M^{-1} \cdot cm^{-1}$) for ferro-minus ferricytochrome *c*. Methyl red reductase activity was determined with a procedure modified from Huang *et al.* (2). The reaction mixture contained 800 ng of enzyme and 0.5 mM methyl red in a final volume of 1 ml. The product, *N,N*-dimethyl-*p*-phenylenediamine, was measured fluorometrically after reaction with fluorescamine.

Electrophoretic and Immunoblot Methods. SDS/10% polyacrylamide gel electrophoresis was performed according to Laemmli (18). Immunoblot analysis was carried out by a modified method of Towbin *et al.* (9). For nondenaturing polyacrylamide gel electrophoresis, the samples were prepared in 50 mM Tris·HCl (pH 6.8) without boiling, and electrophoresis was performed in the absence of SDS and the reducing agent.

RESULTS

Expression and Purification of Heterodimer Enzyme. In comparison to our original NQOR expression system (12), the combined expression levels of NQOR and H194A from the new construct were higher as determined by immunoblot analysis of the cell-free extracts (Fig. 1*A*). When the cytosol of the original expression system was applied onto the Ni-NTA column, most of the NQOR protein did not bind the column and the adsorbed protein in the column was eluted with the wash buffer (data not shown). For the purification of the heterodimer (H194A/HNQOR) from the new construct, the cytosol was applied onto a Ni-NTA column, washed extensively, and eluted with various concentrations of imidazole. The identities of the peptides were determined by SDS/polyacrylamide gel electrophoresis and immunoblot analyses. The homodimer of H194A was in the flowthrough, the heterodimer of H194A/HNQOR was eluted by 30 mM imidazole, and the homodimer of HNQOR/HNQOR was eluted by 40 mM imidazole (Fig. 1 *B* and *C*). This conclusion was also confirmed by nondenaturing polyacrylamide gel electrophoresis, which indicated that the H194A/HNQOR protein was apparently homogeneous, with one band (Fig. 1*D*).

During the purification of H194A/HNQOR, we observed that equal amounts of two polypeptides, H194A and HNQOR, were expressed. The ratio of the three dimers, H194A/H194A,

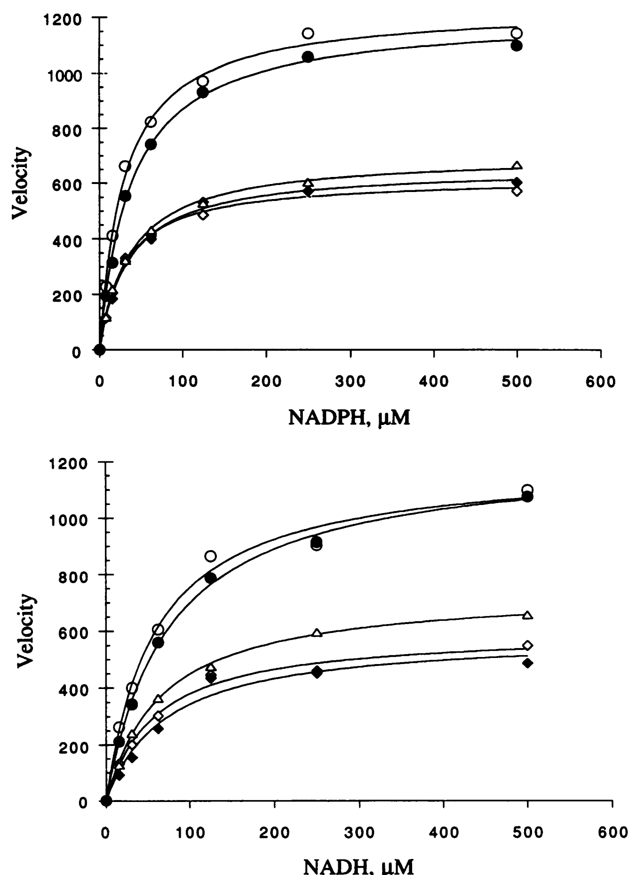

FIG. 2. Reduction of DCIP by heterodimer and homodimers of NQOR with NADPH (*Upper*) and NADH (*Lower*) as the electron donors. ○, NQOR; ●, HNQOR/HNQOR; ◆, H194A/HNQOR; ◇, H194A plus NQOR; △, the predicted curve obtained from the theoretical calculation based on the Michaelis–Menten equation using the sum of $V_{max}/2$ of NQOR and H194A as known parameters. Data for H194A could not be shown due to the higher K_m values. The kinetic studies were carried out in an assay mixture containing 40 μM DCIP and a concentration range of 8–500 μM NADH or NADPH as the electron donors at pH 7.5.

H194A/HNQOR, and HNQOR/HNQOR, was approximately 1:2:1 as estimated by immunoblot analysis (data not shown).

DCIP as an Electron Acceptor. With DCIP as an electron acceptor, HNQOR/HNQOR had $K_{m(NADPH)}$ and $K_{m(NADH)}$ values slightly higher than the corresponding values for NQOR, and k_{cat} values were nearly identical to those of NQOR (Fig. 2 and Table 1). The $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of H194A were 24- and 4-fold higher than the corresponding values of NQOR, and k_{cat} values were 30% and 40% those of NQOR, respectively. The mutation decreased the k_{cat}/K_m values to 1% and 10% of the wild-type enzyme with NADPH and NADH as electron donors, respectively. The $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of H194A/HNQOR were approximately the same as the corresponding values of the HNQOR/HNQOR homodimer, but k_{cat} values were only 50% of those of HNQOR/HNQOR or NQOR. The results from H194A/HNQOR were identical to those from a mixture of H194A and NQOR and close to the predicted kinetics of the sum of the NQOR subunit and the H194A subunit of the heterodimer (Fig. 2 and Table 1).

Menadione as an Electron Acceptor. With menadione as an electron acceptor, the K_m and k_{cat} values of HNQOR/HNQOR for NADPH and NADH were almost the same as those of NQOR (data not shown). The $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of H194A were 26- and 4-fold higher, respectively, than those of NQOR, and k_{cat} values were 70% and 30% of the corresponding values for NQOR. The $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of H194A/HNQOR were not significantly different from those of NQOR and HNQOR/HNQOR. The k_{cat} values of H194A/HNQOR were 50% of those of HNQOR/HNQOR or NQOR and were similar to the values obtained from a mixture of H194A and NQOR. The k_{cat} values were comparable to the corresponding values for the mixture of H194A and NQOR.

Methyl Red as an Electron Acceptor. In comparison to those with two-electron acceptors, the $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of NQOR with the four-electron acceptor methyl red as a substrate were 10- to 20-fold higher. Apparently, the interaction of methyl red with the enzyme also affected the interaction between the enzyme and NAD(P)H, suggesting that the kinetics deviated from a pure ping–pong mechanism as was elucidated from studies with quinones (4). The $K_{m(NADPH)}$ and $K_{m(NADH)}$ values of HNQOR/HNQOR were 3- and 2-fold higher than the corresponding values for NQOR, but k_{cat} values were almost the same as those of NQOR. With H194A, the $K_{m(NADPH)}$ and $K_{m(NADH)}$ values were 22- and 9.5-fold higher than the corresponding values for NQOR, and k_{cat} values were about 50% those of NQOR. With the heterodimer H194A/HNQOR, the $K_{m(NADPH)}$ and $K_{m(NADH)}$ values were 23- and 8-fold higher and k_{cat} values were 40–50% of corresponding values for NQOR (Fig. 3 and Table 2). These data were similar to those for the homodimer H194A mutant enzyme.

DISCUSSION

In the present study, H194A and wild-type HNQOR polypeptides were expressed under the control of one promoter. The

Table 1. Kinetic parameters of homodimeric and heterodimeric enzymes for NAD(P)H with DCIP as a substrate

Electron donor	Enzyme	K_m, μM	k_{cat}, $min^{-1} \times 10^{-3}$	k_{cat}/K_m, $\mu M^{-1} \cdot min^{-1}$
NADPH	NQOR	30 ± 2[a] (1.0)	75 ± 2[a] (1.0)	2500 (1.00)
	HNQOR/HNQOR	39 ± 2[a] (1.3)	74 ± 1[a] (1.0)	1897 (0.75)
	H194A	722 ± 110[b] (24)	32 ± 5[b] (0.4)	36 (0.01)
	H194A/HNQOR	36 ± 3[a] (1.2)	39 ± 1[b] (0.5)	1083 (0.43)
	H194A+NQOR	30 ± 2[a] (1.0)	38 ± 1[b] (0.5)	1266 (0.50)
NADH	NQOR	57 ± 10[a] (1.0)	73 ± 4[a] (1.0)	1280 (1.00)
	HNQOR/HNQOR	85 ± 11[a] (1.2)	75 ± 4[a] (1.0)	882 (0.68)
	H194A	254 ± 43[b] (4.4)	26 ± 5[b] (0.3)	126 (0.10)
	H194A/HNQOR	70 ± 15[a] (1.1)	36 ± 3[b] (0.5)	514 (0.40)
	H194A+NQOR	55 ± 11[a] (1.0)	37 ± 3[b] (0.5)	672 (0.52)

The kinetic parameters were calculated from the data in Fig. 2 by using the Michaelis–Menten equation and are shown as mean ± SD ($n = 3$). Numbers in parentheses represent relative values as compared with those of wild-type NQOR. Each value was the average of three sets of experiments. The means with different superscripts in the same column are significantly different ($P < 0.05$) from each other as determined by analysis of variance followed by the Newman–Keuls range test.

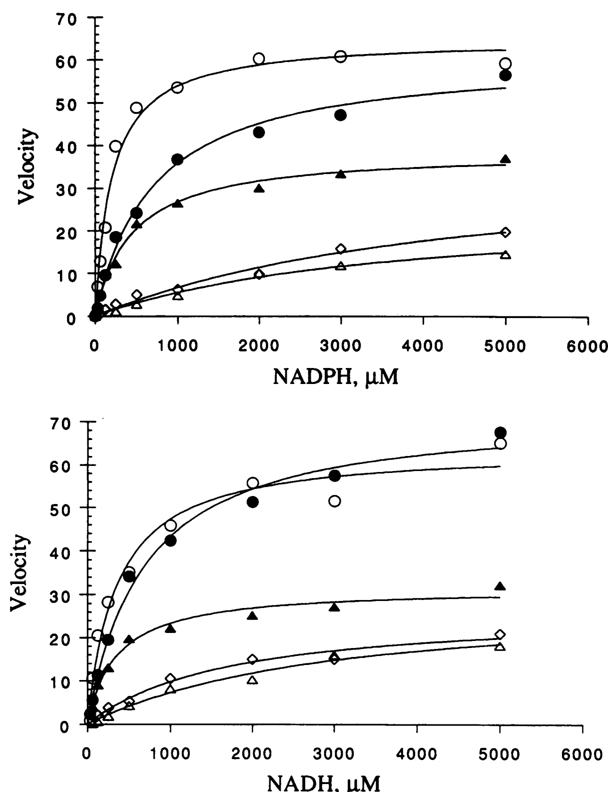

FIG. 3. Reduction of methyl red by heterodimer and homodimers with NADPH (*Upper*) and NADH (*Lower*) as the electron donors. ○, NQOR; ●, HNQOR/HNQOR; △, H194A; ◇, H194A/HNQOR; ▲, H194A plus NQOR. Kinetic studies for NAD(P)H of purified enzymes were performed in an assay mixture containing 100 μM methyl red as the substrate and a concentration range of 0.04–5 mM NAD(P)H at pH 6.1.

transcript is expected to be one mRNA containing two ribosome binding sites, one for the H194A polypeptide and the other for the HNQOR polypeptide. The observation that three species of dimers—H194A/H194A, H194A/HNQOR, and HNQOR/HNQOR—were produced at the ratio of 1:2:1 suggested that both polypeptides were synthesized at the same rate and that neither the site of mutation nor the His_6 tag significantly affected the dimerization process. Furthermore, these three species of dimers were separated successfully from each other according to their affinities for a Ni-NTA column. With the purified H194A/HNQOR heterodimer, the subunit functions of NQOR can be elucidated by kinetic studies. If the

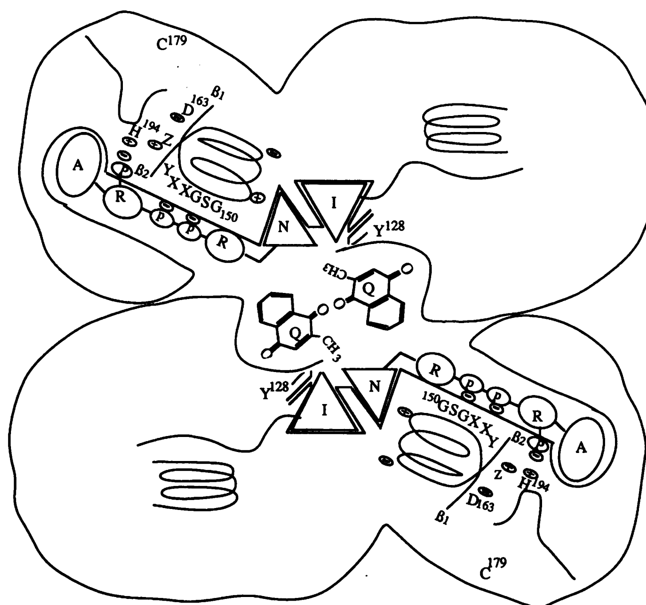

FIG. 4. Proposed dimeric model of NQOR. Two identical monomers in the opposite orientation form a dimer. The quinone binding sites [occupied by menadione (Q) in the figure] of the two subunits are proposed to be close to each other. The azo dye binding site is suggested to be fitted into the quinone binding site at the interface of the two subunits and close to two FAD molecules. Other features of the model are similar to those described previously (8, 10). The glycine-rich region, forming a turn between β_1 and α-helix in the $\beta\alpha\beta$ motif, is the binding site for the pyrophosphate group of NAD(P)H. His-194 (H^{194}) interacts directly with the 2′-phosphate group of NADPH by a charge–charge interaction. Asp-163 (D^{163}) is proposed to be in a position close to the α helix affecting its polarity and interacting with a hypothetical positively charged residue (Z^+). A hypothetical isoalloxazine (I) binding site is shown; the pyrophosphate (not shown) is proposed to bind to a second $\beta\alpha\beta$ structure. Tyr-128 (Y^{128}), which affects the fluorescence of FAD, and Cys-179 (C^{179}), which is important for the stability of NQOR, are also shown.

subunits function independently, H194A/HNQOR should have the sum of the activities of the two subunits. Since H194A has very low catalytic efficiency (Table 1), H194A/HNQOR is expected to have the same K_m and about 50% the k_{cat} of wild-type NQOR. This concept is consistent with the experi-

Table 2. Kinetic parameters of homodimer and heterodimer enzymes for NAD(P)H with methyl red as a substrate

Electron donor	Enzyme	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, μM^{-1}·min^{-1}
NADPH	NQOR	204 ± 25[a] (1.0)	3965 ± 111[a] (1.0)	19.4 (1.00)
	HNQOR/HNQOR	720 ± 101[b] (3.6)	3721 ± 150[a] (0.9)	5.2 (0.30)
	H194A	4257 ± 1132[c] (22)	1525 ± 625[b] (0.4)	0.4 (0.02)
	H194A/HNQOR	4765 ± 1453[c] (23)	1952 ± 317[b] (0.5)	0.4 (0.02)
	H194A+NQOR	356 ± 51[a] (1.1)	1891 ± 73[b] (0.6)	5.3 (0.30)
NADH	NQOR	330 ± 72[a] (1.0)	3886 ± 201[a] (1.0)	11.8 (1.00)
	HNQOR/HNQOR	680 ± 122[b] (2.1)	4514 ± 250[a] (1.2)	6.6 (0.56)
	H194A	3121 ± 803[c] (9.5)	1830 ± 244[b] (0.5)	0.6 (0.05)
	H194A/HNQOR	2749 ± 385[c] (8.3)	1586 ± 146[b] (0.4)	0.6 (0.05)
	H194A+NQOR	490 ± 111[a] (1.6)	2051 ± 200[b] (0.6)	4.6 (0.39)

The kinetic parameters were calculated from the data in Fig. 3 by using the Michaelis–Menten equation and are shown as mean ± SD. Numbers in parentheses represent relative values as compared with those of wild-type NQOR. Each value was the average of three to five sets of experiments. The means with different superscripts in the same column are significantly different ($P < 0.05$) from each other as determined by analysis of variance followed by the Newman–Keuls range test.

mental results with the two-electron acceptors DCIP and menadione. If the subunits of NQOR are functionally dependent, the heterodimer enzyme is expected to behave like the less efficient H194A protein. This mode of action was found in the reduction of methyl red by H194A/NQOR. It appears that the reduction of methyl red requires the transfer of a pair of electrons from each subunit of NQOR. With H194A/HNQOR, the second pair of electrons will have to come from the mutant subunit, H194A, and this becomes the rate-limiting step. Thus, the kinetics of methyl red reduction by H194A/HNQOR were similar to that of the mutant H194A homodimer (Table 2 and Fig. 3). This result suggests that the subunits of NQOR function dependently for methyl red and perhaps also for other four-electron acceptors.

Based on the present results, a model for the dimeric structure of NQOR is proposed as shown in Fig. 4. It builds on the features of the previously proposed NAD(P)H binding sites (8, 10) and suggests that two quinone molecules are reduced to hydroquinones by accepting electrons from NAD(P)H at both subunits (Fig. 4). The methyl red binds at the interface of the two subunits and reaches to the substrate binding sites of both subunits in order to receive four electrons from the two subunits (not shown in the figure). We propose that the subunits of NQOR are functionally independent for two-electron acceptors but dependent for methyl red, a four-electron acceptor. The same conclusion was also reached by similar studies with the heterodimer of another mutant, H194D (data not shown).

The approach we developed to generate and purify the site-directed mutant/wild-type heterodimer may have general applications in the studies of subunit functions of dimeric or oligomeric proteins. The expression of two polypeptides under the control of one promoter permits optimal production of heterodimers. The use of the His_6 tag facilitates the separation of the heterodimer from the homodimer. This approach may be used in the following types of applications. (*i*) It can be used to study the effect of dimer formation on the stability of dimeric enzymes, for example, by expressing a heterodimer containing a wild-type subunit of NQOR and a mutant subunit, such as mutant C179A, which is known to be less stable than the wild-type NQOR (10). With the present approach, it is possible to determine in the heterodimer, C179A/HNQOR, whether the instability of the C179 mutant would affect the stability of the other subunit. (*ii*) It can be used to study the involvement of various domains in subunit interaction. For NQOR as an example, heterodimers could be made from one wild-type NQOR subunit and another subunit His_6-tagged either at the amino terminus or at the carboxyl terminus. By stepwise deletions of either the carboxyl or the amino terminus of the tagged subunit, the minimum structural requirements for dimeric function (i.e., both subunits are functional) or monomeric function (i.e., the domains in the modified subunit are required to maintain the wild-type subunit in a functional conformation) could be determined. (*iii*) With allosteric enzymes, mutation at the allosteric site of one subunit but not at other subunits may reveal interesting properties concerning the cooperativity among the subunits.

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