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Evidence for three separate electron flow pathways through Complex I: an inhibitor study

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Abstract

The mammalian mitochondrial electron transport chain catalyzes the oxidation of NADH at pH 8.0 and pH 6.5, and the oxidation of NADPH at pH 6.5. The pH-dependencies of the rate of steady-state oxidation of NADPH and NADH by Complex I as well as by its flavoprotein fraction have been extensively studied by the laboratory of Hatefi. One model to explain these pH-dependent oxidations was proposed by Bakker and Albracht (Biochim. Biophys. Acta 850 (1986) 413–422 and 423–428, modified by Van Belzen and Albracht (Biochim. Biophys Acta 974 (1989) 311–320), which predicts that Complex I is a heterodimer with protomer B, containing FMN and Fe-S clusters 1–4 in stiochiometric amounts, catalyzing NADH oxidation at pH 8, and Protomer A, containing FMN and Fe-S clusters 2, 4, catalyzing NAD(P)H oxidation at pH 6.5. A pH-dependent transfer of electrons from protomer A Fe-S clusters 2, 4 to protomer B Fe-S clusters 2, 4 is an obligate step in the oxidation of NAD(P)H at low pH. Strict interpretation of this model allows for only three types of inhibitor: one which inhibits all three oxidase activities (type 1); one which inhibits NADH oxidase, pH 8.0 (type 4) and a third which inhibits NAD(P)H oxidase, pH 6.5 (type 5). Another possibility is that there are three separate pathways of oxidation of NAD(P)H, which would allow for a total of seven different types of inhibitor, e.g., the three types above plus type 2 inhibiting NADH oxidase pH 8.0 and pH 6.5; type 3 inhibiting NADH oxidase pH 8.0, and NADPH oxidase pH 6.5; type 6 inhibiting NADH oxidase pH 6.5; and type 7 inhibiting NADPH oxidase pH 6.5. Using a series of thirteen inhibitors of Complex I activity and the chemical modification reagent ethoxyformic anhydride (EFA), four different inhibitor types were found: seven inhibitors of type 1, four inhibitors of type 2, one inhibitor of type 3 and one inhibitor of type 4. Treatment of submitochondrial particles (SMP) with EFA abolished NADH-dependent reduction of coenzyme Q at both pH 8.0 and 6.5, while inhibiting NADPH-dependent reduction of coenzyme Q at pH 6.5 by only 30%. These results do not support the heterodimer model of Complex I electron transport of Bakker and Albracht, but do support three separate electron flow pathways through complex 1 from reduced pyridine nucleotides to coenzyme Q. A new model of electron flow through Complex I based on these finding is proposed.

Keywords: Mitochondrion; NADH oxidase; NADPH oxidase; Electron transport chain

1. Introduction

The pathway of electron transport from NADH to oxygen (NADH oxidase (pH 8.0)) has been investigated in great detail in both mitochondria and *Paracoccus denitrificans* (see Refs. [1–3] for reviews of the NADH-ubiquinone reductase portion of the oxidase activity). Although it has been known for some time that mitochondria catalyze an NADH (pH 8.0) oxidase and an NAD(P)H oxidase activity at pH 6.5, much less is known about this latter oxidase activity, although early studies from the laboratory of Hatefi [4–7], indicated that the initial steps of NADPH oxidation were catalyzed by Complex I and specifically the flavoprotein fraction and that this activity involved reduction of FMN, iron-sulfur cluster 2 and perhaps iron-

Abbreviations: SMP, submitochondrial particles; DMSO, dimethylsulfoxide; S13, 3,3′-dimethylthiacarbocyanine iodide; S23, 3,3′-diethylthiacarbocyanine iodide; S25, 3,3′-diethylthiadicarbocyanine iodide; S33, 3,3′-dipropylthiacarbocyanine iodide; S43, 3,3′-dibutylthiacarbocyanine iodide; S53, 3,3′-dipentylthiacarbocyanine iodide; S25, 3,3′-diethylthiadicarbocyanine iodide; HIDC, 1,1′,3,3,3′,3′-hexamethylindodicarbocyanine iodide; HITC, 1,1′,3,3,3′,3′-hexamethylindotricarbocyanine iodide; DiOC2(3), 3,3′-diethyloxacarbocyanine; DiOC5(3), 3,3′-dipentyloxacarbocyanine; DiOC6(3), 3,3′-dihexyloxacarbocyanine; DiOC2(4), 3,3′-diethyloxadicarbocyanine; DECA, dequalinium chloride; Di-16-ASQ, 4-(*p*-dihexadecylaminostryl)-*N*-methylquinolinium iodide; DBQ, 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone; EFA, ethoxyformic anhydride.

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sulfur clusters 3 and 4. Kinetic analysis by Bakker and Albracht, later reexamined by Van Belzen and Albracht, of electron flow through Complex I, led these workers to proposed a heterodimeric model of Complex I [8–10] in which protomer B contains FMN and Fe-S clusters 1–4 in stiochiometric amounts and oxidizes NADH at pH 8.0, but cannot react with NADPH. Protomer A contains FMN and Fe-S clusters 2, 3 and 4 and catalyzes NAD(P)H oxidation at pH 6.5. A pH-dependent electron transfer from protomer A to B between Fe-S clusters 2 and 4 of protomer A to Fe-S clusters 2 and 4 of protomer B is a necessary step in the oxidation of NAD(P)H and reduction of ubiquinone at the lower pH. Thus this model of electron flow envisions two different routes of electron transport depending upon the electron source and/or pH, converging at Fe-S clusters 4 and 2 of protomer B to allow NAD(P)H oxidase activity to occur at pH 6.5 as well as NADH oxidase at pH 8.0. While this hypothesis is intriguing, except for the report by Glinn et al. [11] that Complex I has two distinct pyridine nucleotide binding sites, a low-affinity NADH and rhein binding site and a high-affinity NAD(P)H binding site that does not bind rhein, there has been little further substantiating evidence. As pointed out by Walker in a recent review [1], the lack of progress in the area of the electron transport pathway through Complex I is hampered both by the inability to resolve intramolecular electron transfers between Fe-S clusters by EPR and by the lack of inhibitors which block at specific intermediate sites.

For the past several years, workers in this laboratory have been investigating new inhibitors of NADH-ubiquinone reductase activity in bovine heart submitochondrial particles and *P. denitrificans* membrane vesicles [12–15]. We have discovered a number of inhibitory compounds which appear to act at or near the rotenone-binding site of NADH-ubiquinone reductase and at least one appears to act at a different site.

Using these new inhibitors, along with the two classical inhibitors, rotenone and piericidin A, erythrosin 5′-iodoacetamide, reported to bind at the ubiquinone binding site of Complex I [16], and ethoxyformic anhydride, a chemical modification reagent reported to inhibit NADH-dependent reduction of coenzyme Q by ethoxyformylating histidine and tyrosine residues of Complex I [17], we tested the Bakker and Albracht model as well as a model which allows for separate electron flow pathways for NADH

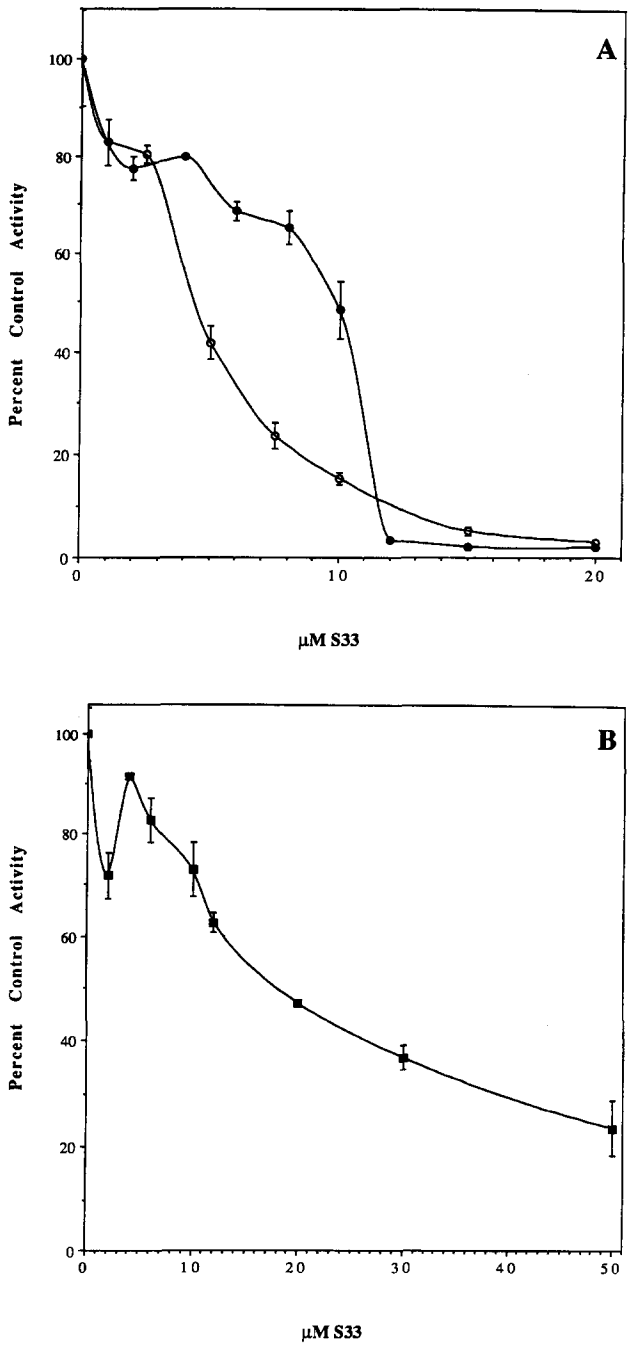

Fig. 1. Effect of S33 on submitochondrial particle NADH oxidase (pH 8.0) NADH oxidase (pH 6.5) (A), and NADPH oxidase (pH 6.5) (B) activity. Assays were performed as described in Materials and methods. NADH oxidase assays (pH 8.0 and 6.5) contained 0.05 mg/ml SMP protein; NADPH oxidase assays contained 1 mg/ml SMP protein. Key: ●, NADH (pH 8.0); ○, NADH (pH 6.5); ■, NADPH (pH 6.5). Control specific activities (units/mg protein) NADH (pH 8.0), NADH (pH 6.5) and NADPH (pH 6.5) were 1088, 957 and 15, respectively.

Table 1
Inhibitor types based upon effect on NADH oxidase (pH 8.0), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5)

Inhibitor type	Effect [a]		
	NADH oxidase (pH 8.0)	NADH oxidase (pH 6.5)	NADPH oxidase (pH 6.5)
1	+	+	+
2	+	+	−
3	+	−	+
4	+	−	−
5	−	+	+
6	−	+	−
7	−	−	+

[a] A+ indicates inhibition, a− indicates no inhibition (defined as two or more successive concentrations yielding < 50% control activity).

oxidation at pH 8.0, NADH oxidation at pH 6.5 and NADPH oxidation at pH 6.5. This latter model would allow for a total of seven different inhibitor types shown in Table 1. Strict interpretation of the Bakker and Albracht model would allow for only three types of inhibitor, i.e., types 1, 4 and 5. In the Bakker and Albracht model, type 1 inhibitors would block electron transport between protomer B Fe-S clusters, 4, 2 to coenzyme Q; type 4 inhibitors would block electron transfer between at Fe-S clusters 1 and 3, while type 5 inhibitors would block the pH-dependent electron transfer between protomer A Fe-S clusters 4, 2 and protomer B Fe-S clusters 4, 2.

2. Materials and methods

2.1. Preparations and assays

SMP were prepared from bovine hearts as described previously [18]. NADH oxidase (pH 8.0) activity was determined spectrophotometrically at 25° C as described previously [12]. NADH oxidase (pH 6.5) activity was determined spectrophotometrically at 25° C in a 1 ml assay system which was identical to the NADH oxidase (pH 8.0) system except that the sodium phosphate buffer was pH 6.5. NADPH oxidase (pH 6.5) activity was determined spectrophotometrically at 25° C in a 1 ml assay system containing 2 mM KMops (pH 6.5), 150 mM KCl ± 200 μM rhein [11]. In our hands the activity was identical in the absence and presence of rhein. With respect to NADPH oxidase activity, (a) treatment SMP for 1 h at 37° C with trypsin (1 mg/ml) to destroy pyridine dinucleotide transhydrogenase activity, or (b) inclusion of 1 μM palmitoyl CoA (to inhibit pyridine dinucleotide transhydrogenase activity) in the assay system [19] had no effect (1% or less) on the measurable NADPH oxidase activity. NAD(P)H-ubiquinone reductase activity was determined spectrophotometrically in a 1 ml assay system described above containing 125 μM DBQ, 50 μM antimycin A and 10 mM NaN_3. Treatment of SMP with ethoxyformic anhydride (final concentration 6.9 mM) was as described by Vik and Hatefi [17] except that SMP was used instead of Complex I. Following incubation with EFA for 20 min at 0° C, SMP were pelleted by centrifugation at 200 000 × *g* for 30 min at 4° C, then resuspended in 10 mM KMops (pH 7.5), containing 250 mM sucrose to a protein concentration of 10 mg/ml. Assays with inhibitors were performed by adding buffer, deionized water, reduced pyridine nucleotide (DBQ, antimycin A, NaN_3 where applicable), to the cuvet, mixing, then adding the inhibitor, mixing again and finally adding SMP to start the reaction. To ensure that steady-state rates were being measured, the enzymatic rate was followed for up to 10 min. For all inhibitors except rotenone, which is a slow binding inhibitor, the steady-state rate was observed as early as 30 s. Steady-state rates were obtained with rotenone at about 1 min and the linear rate was followed for 10 min. In our hands, the time to attain linearity was independent of enzyme concentration. After each assay, cuvets were rinsed twice with deionized water, six times with 95% (w/v) ethanol and six times with deionized water. In all cases

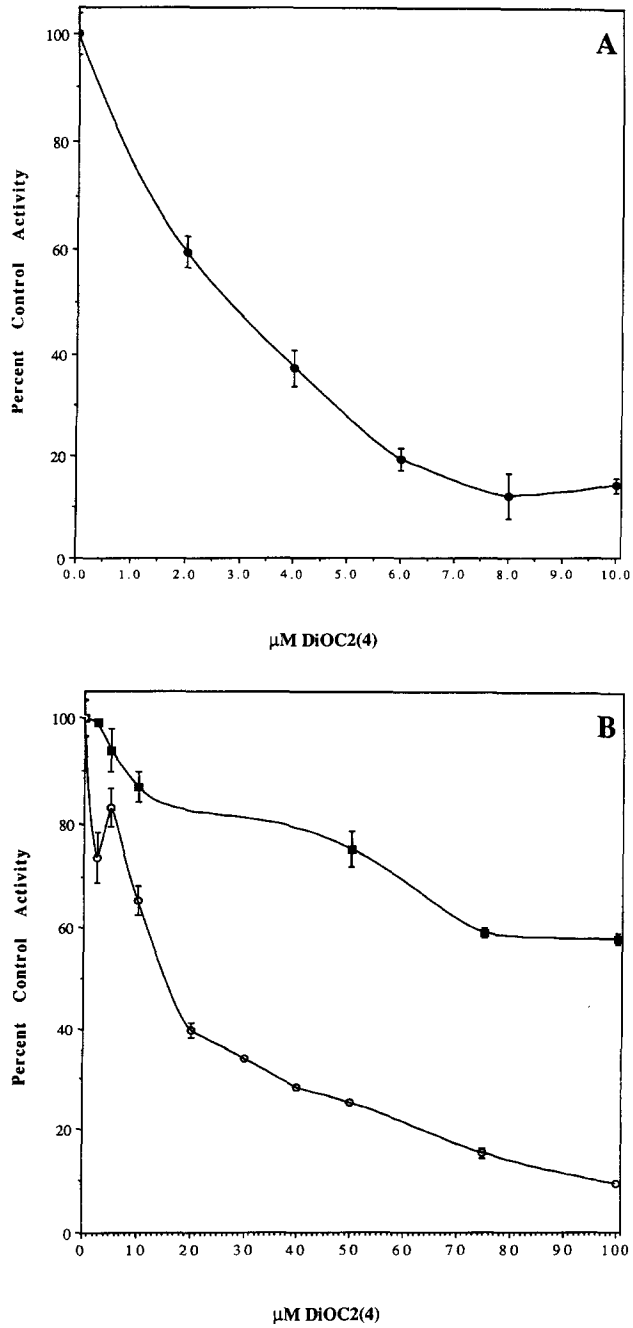

Fig. 2. Effect of DiOC2(4) on submitochondrial particle NADH oxidase (pH 8.0) (A), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5) (B) activity. Assays were performed as described in Materials and Methods. NADH oxidase assays (pH 8.0 and 6.5) contained 0.05 mg/ml SMP protein; NADPH oxidase assays contained 1 mg/ml SMP protein. Key: ●, NADH (pH 8.0); ○, NADH (pH 6.5); ■, NADPH (pH 6.5). Control specific activities (units/mg protein) NADH (pH 8.0), NADH (pH 6.5) and NADPH (pH 6.5) were 1088, 957 and 15, respectively.

Table 2
I_{50} of type 1 inhibitors for NADH oxidase (pH 8.0), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5)

Inhibitor	I_{50} [a]		
	NADH oxidase (pH 8.0)	NADH oxidase (pH 6.5)	NADPH oxidase (pH 6.5)
Rotenone	0.016 (0.32)	0.025 (0.5)	0.03 (0.03)
Piericidin A	0.014 (0.28)	0.065 (1.3)	0.035 (0.035)
Erythrosin 5′-iodoacetamide	0.02 (0.4)	20.5 (410)	41.5 (41.5)
HIDC	0.05 (1.0)	10.3 (206)	11.5 (11.5)
HITC	1.5 (30)	10.5 (210)	2.0 (2.0)
S33	10 (200)	4.5 (90)	19 (19)
S43	10.5 (210)	3.0 (60)	55 (55)

[a] I_{50} in μM; numbers in parentheses are nmol/mg SMP protein.

assays were performed in triplicate and data presented represent the mean ± S.E. Protein concentration was determined by the biuret reaction as described by Jacobs et al. [20] using crystalline bovine serum albumin as a standard.

2.2. Materials

Piericidin A was a gift from Dr. Simon DeVries, University of Amsterdam. NADPH, rotenone, HIDC, HITC, DECA, Mops, DBQ, antimycin A, trypsin, palmitoyl CoA and DMSO were purchased from Sigma. S13, S23, S25, S33, S43 and S53 were obtained from Kodak. Di-16-ASQ, DiOC2(3), DiOC5(3), DiOC6(3) and DiOC2(4) and erythrosin 5′-iodoacetamide were purchased from Molecular Probes. Rhein was obtained from Aldrich. All other chemicals were of reagent-grade quality.

All inhibitors, except piericidin A, which was dissolved in ethanol, were dissolved in DMSO at concentrations at or near 10 mM. DBQ was dissolved in ethanol at a concentration of 2.5 mM. Inhibitors could be stored wrapped in aluminum foil at −20° C for 1 week.

3. Results

3.1. Titration of SMP NADH oxidase (pH 8.0), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5) with inhibitors

Inhibitors were chosen after first testing to eliminate the possibility that there was a pH effect on the compound, by determining the spectra in the UV and visible region of each compound in the buffer systems used in the study. Any compound which showed dramatic changes (appearance or disappearance of absorbance peaks) was eliminated. The thiacarbocyanine, S25, showed significant spectral changes and was eliminated from this study (data not shown). A total of sixteen inhibitory compounds showed no significant spectral changes. Second, inhibitors were tested for their ability to inhibit (> 90% inhibition) the SMP NADH-dependent reduction of DBQ at pH 8.0. Three inhibitors, Di-16-ASQ, S13 and DiOC5(3) were eliminated from the study, based on their inability to inhibit (< 50% inhibition) DBQ reduction (data not shown), leaving thirteen compounds to be tested. These are rotenone, piericidin A, HIDC, HITC, DECA, erythrosin 5′-iodoacetamide, S23, S33, S43, S53, DiOC2(3), DiOC2(4) and DiOC6(3). In this laboratory we have demonstrated specific inhibition of mitochondrial and *P. denitrificans* NADH-ubiquinone reductase activity by DECA [12], HIDC and HITC [13], DIOC2(3), DIOC5(3), DIOC6(3) and DIOC2(4) [15], and have described the effect of the thiacarbocyanines S13, S23, S33, S43 and S53 on mitochondrial NADH-ubiquinone reductase [14]. Ahmed and Krishnamoorthy have reported that erythrosin 5′-iodoacetamide binds to mitochondrial NADH-ubiquinone reductase at the ubiquinone binding site [16]. Titrations of the three oxidase activities were performed over a 50–100-fold range of concentration (except for rotenone and piericidin A, where the range was 20-fold) and, for the purposes of this work, inhibition is defined as two or more successive concentrations of the inhibitory compound which yielded more than 50% of control activity.

Seven of the thirteen compounds inhibited all three oxidase activities (type 1 inhibitors-see Table 1). These are rotenone, piericidin A, erythrosin 5′-iodoacetamide, HIDC, HITC, S33 and S43. Data for one of these type 1 inhibitors, S33, are shown in Fig. 1A,B. I_{50} for each of the seven type 1 inhibitors is given in Table 2. We had previously reported that neither S33 nor S43 inhibited mammalian mitochondrial NADH oxidase activity at pH

Table 3
I_{50} of type 2 inhibitors for NADH oxidase (pH 8.0) and NADH oxidase (pH 6.5)

Inhibitor	I_{50} [a]	
	NADH oxidase (pH 8.0)	NADH oxidase (pH 6.5)
DiOC2(4)	2.8 (56)	10 (200)
DiOC6(3)	0.08 (1.6)	17 (340)
S23	1.2 (24)	48 (960)
S53	10.2 (204)	2.1 (42)

[a] I_{50} in μM; numbers in parentheses are nmol/mg SMP protein.

8.0 based upon a titration from 1 to 7 μM [14]. Titrations of both of these compounds was stopped at 7 μM, since precipitation was observed in the assay system above this concentration. Using the mixing regimen described in 2.1 above, there was no precipitation, and inhibition of NADH oxidase (pH 8.0) was demonstrated with the I_{50} of both of these two thiacarbocyanines being about 10 μM. With the exception of rotenone, S33 and S43, the I_{50} for NADH oxidase (pH 8.0) was lower than that for either NADH or NADPH oxidase (pH 6.5). These results are consistent with both the Bakker and Albrach heterodimer model and the three separate pathways model.

Four of the thirteen compounds inhibited NADH oxidase at pH 8.0 and 6.5 but did not inhibit NADPH oxidase (pH 6.5) (type 2 inhibitors –see Table 1). These are DiOC6(3), DiOC2(4), S23 and S53. Data for DiOC2(4) are shown in Fig. 2A,B. I_{50} for each of the four type 2 inhibitors is given in Table 3. As with S33 and S43, we had also previously reported that S53 was not an inhibitor of NADH oxidase (pH 8.0) [14], but with the mixing regimen in 2.1, we were able to extend the titration range to observe inhibition. With the exception of S53, the I_{50} for NADH oxidase (pH 8.0) is lower than for NADH oxidase (pH 6.5). These results are not consistent with the Bakker and Albracht heterodimer model, but are consistent with the three separate pathways model.

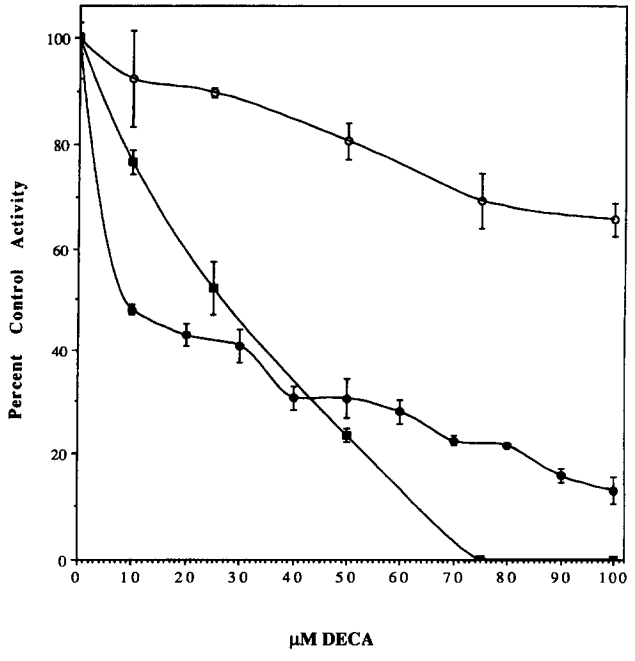

Fig. 3. Effect of DECA on submitochondrial particle NADH oxidase (pH 8.0), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5) activity. Assays were performed as described in Materials and methods. NADH oxidase assays (pH 8.0 and 6.5) contained 0.05 mg/ml SMP protein; NADPH oxidase assays contained 1 mg/ml SMP protein. Key ○, NADH (pH 8.0); ○, NADH (pH 6.5); ■, NADPH (pH 6.5). Control specific activities (units/mg protein) NADH (pH 8.0), NADH (pH 6.5) and NADPH (pH 6.5) were 1088, 957 and 15, respectively.

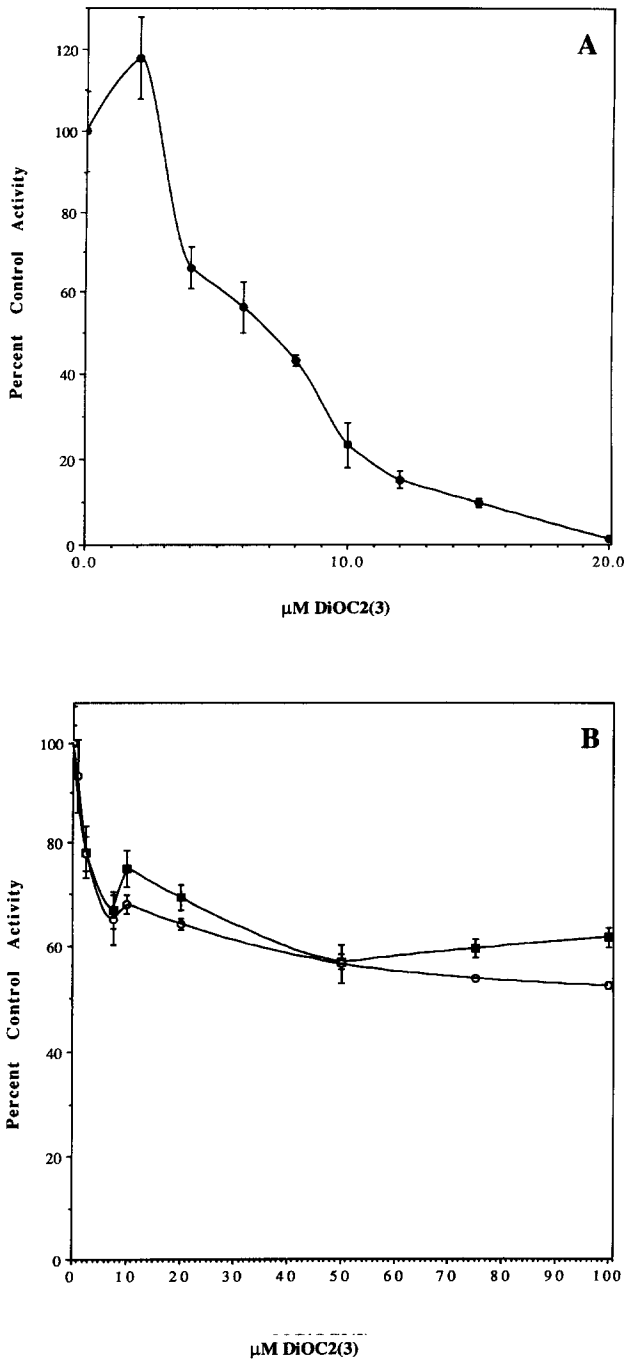

Fig. 4. Effect of DiOC2(3) on submitochondrial particle NADH oxidase (pH 8.0) (A), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5) (B) activity. Assays were performed as described in Materials and methods. NADH oxidase assays (pH 8.0 and 6.5) contained 0.05 mg/ml SMP protein; NADPH oxidase assays contained 1 mg/ml SMP protein. Key: ●, NADH (pH 8.0); ○, NADH (pH 6.5); ■, NADPH (pH 6.5). Control specific activities (units/mg protein) NADH (pH 8.0), NADH (pH 6.5) and NADPH (pH 6.5) were 1088, 957 and 15, respectively.

One of the thirteen compounds, DECA, inhibited NADH oxidase (pH 8.0) and NADPH oxidase (pH 6.5), but did not inhibit NADH oxidase (pH 6.5) (type 3 inhibitor –see Table 1). These results are presented in Fig. 3. The I_{50} for

NADH oxidase (pH 8.0) was 9.5 μM and it was 28 μM for NADPH oxidase (pH 6.5). The I_{50} for NADH oxidase (pH 8.0) is close to the previously reported 11 μM for this activity [12]. As with the type 2 inhibitors, this inhibition pattern by DECA is not consistent with the Bakker and Albracht heterodimer model, but is consistent with the three separate pathways model.

One of the thirteen compounds, DiOC2(3) inhibited NADH oxidase (pH 8.0), but not NADH or NADPH oxidase (pH 6.5) (type 4 inhibitors –see Table 1). These results are presented in Fig. 4A,B. The I_{50} for NADH oxidase (pH 8.0) was 7.3 μM, which closely compares with the 9 μM previously reported for this compound [15]. These results are consistent with either the Bakker and Albracht heterodimer model or the three separate pathways model.

None of the thirteen compounds examined in this study inhibited NADH and NADPH oxidase (pH 6.5) only (type 5 inhibitors –see Table 1), inhibited NADH oxidase (pH 6.5) only (type 6 inhibitors –see Table 1), or inhibited NADPH oxidase (pH 6.5) only (type 7 inhibitors –see Table 1).

3.2. Effect of ethoxyformic anhydride on SMP NADH oxidase (pH 8.0), NADH oxidase (pH 6.5) and NADPH oxidase (pH 6.5)

Treatment of SMP with ethoxyformic anhydride [19], as described in 2.1, completely abolished NADH-dependent reduction of DBQ at pH 8.0 and pH 6.5 (100% inhibition), but only resulted in a 30% inhibition of NADPH-dependent reduction of DBQ at pH 6.5. Taken together, these results are not consistent with the Bakker and Albracht heterodimer model, since strict interpretation of the model would predict that compounds which inhibited NADH-dependent reduction of DBQ (pH 6.5) would also inhibit NADPH-dependent reduction of DBQ (pH 6.5). The data, however, are consistent with the three separate pathways model.

4. Discussion

The results presented here do not support the heterodimer model of two separate electron transport pathways through Complex I proposed by Bakker and Albracht, but are consistent with a three separate pathways for the reduction of coenzyme Q by reduced pyridine nucleotides. The fact that neither trypsin treatment nor inclusion of palmitoyl CoA in the assay system had any effect on the NADPH oxidase activity at pH 6.5 indicates that these results cannot be explained on the basis of pyridine dinucleotide transhydrogenase interference, although no NAD^+ was added to the assay systems. Further, care was taken that the effects observed in the study were not due to the differences in pH by eliminating any compound which showed significant changes in visible and ultraviolet spectra, which eliminated one compound from our study. Except for type 4 inhibitors, the other three inhibitor types observed in this study affected activities at both pH 8.0 and 6.5, which argues further that the effects presented here were not due to pH effects on binding of the inhibitory compounds to the Complex I portion of the electron transport chain. In the Bakker and Albracht model, an inhibitor with acts at pH 6.5 should inhibit both NADH and NADPH oxidase at this pH, i.e., inhibit by binding at the same site. The fact that four compounds were found to inhibit NADH oxidase (pH 6.5) but not NADPH oxidase (pH 6.5) (type 2 inhibitors), and one compound was found to inhibit NADPH oxidase (pH 6.5) but not NADH oxidase (pH 6.5) indicates that there are different inhibitory binding sites at this pH rather than one inhibitor site. The effectiveness of the inhibitor as measured by I_{50} may depend on the steady-state rates of the enzyme reaction, which are different for NADH and NADPH (see Fig. 1, for specific activities). Whereas the ratios of specific activities for NADH oxidase (pH 8.0) to NADPH oxidase (pH 6.5) is 72.5, the ratios of I_{50} values for the same two activities (on a nmol/mg SMP protein basis) for type 1 inhibitors (see Table 2) vary from $9.6 \cdot 10^{-3}$ for erythrosin 5'-iodoacetamide to 15 for HITC, and for DECA, a type 3 inhibitor the ratio of the I_{50} values for these same two activities is 6.8. Thus, there is no general correlation of the rates of enzymatic reaction with inhibitor effectiveness, which argues against the present results being a function of differences in enzymatic reaction rates.

The possibility that the results could be due to redox acceptor activity of the compounds tested (or slight impurities which might be present in the samples tested) was eliminated by excluding any compound which did not exhibit more than 90% inhibition of NADH-dependent reduction of DBQ at pH 8.0. This test eliminated three compounds from the present study. Thus, it is highly unlikely that the results presented here can be explained on the basis of pH effects on chemical structure of the inhibitor or its binding site.

Demonstration of four type 2 inhibitor compounds and one type 3 inhibitor compound argue that there are indeed three separate pathways for the reduction of coenzyme Q by reduced pyridine nucleotides through Complex I. These two types of inhibitor are inconsistent with the Bakker and Albracht model. Further, the fact that chemical modification by ethoxyformic anhydride resulted in complete abolition of NADH-dependent reduction of DBQ at pH 8.0 and 6.5, while only inhibiting NADPH-dependent reduction of DBQ at pH 6.5, strengthens the possibility that there are three separate pathways for reduction of coenzyme Q by reduced pyridine nucleotides. The fact that no type 5, 6 or 7 inhibitors were identified in the present study does not imply that they are nonexistent, but rather that a more comprehensive search will be necessary. A large number of compounds have been found to inhibit Complex I

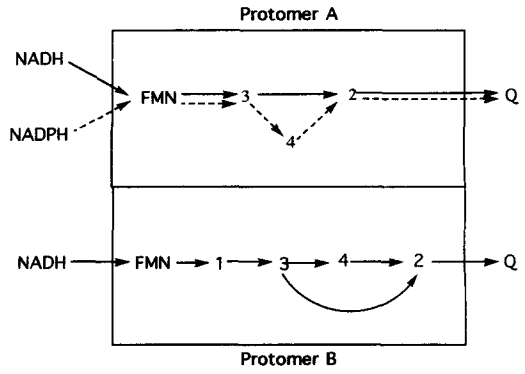

Fig. 5. Schematic representation of the modified heterodimer model of three separate pathways for oxidation of reduced pyridine nucleotides by Complex I. The arrows indicate the proposed pathway of electron flow within the enzyme.

activity and only 13 were examined here. Finding type 7 inhibitors will require looking for compounds which inhibit NADPH-linked activities, as well as an empirical search.

As a working hypothesis, we propose a modified heterodimer model which could explain the existence of three separate pathways and account for the action of the seven different inhibitor types. This model is presented in Fig. 5. In this model, both protomers A and B would be able to reduce coenzyme Q in a pH-dependent manner. Type 1 inhibitors would block the pH-independent reduction of coenzyme Q by reduced Fe-S cluster 2 in either protomer. Type 2 inhibitors would inhibit the pH-independent direct reduction of Fe-S cluster 2 by reduced Fe-S cluster 3 in either protomer, bypassing Fe-S cluster 4 in protomer B. Type 3 inhibitors would block the pH-independent reduction of Fe-S cluster 2 by reduced Fe-S cluster 4 in either protomer. Type 4 inhibitors would block reduction of Fe-S cluster 3 by reduced Fe-S cluster 1 in protomer B. Type 5 inhibitors would block the low pH-dependent reduction of Fe-S cluster 2 by either reduced Fe-S clusters 3 or 4 in protomer A. Type 6 inhibitors would block the low pH-dependent direct reduction of Fe-S cluster 2 by Fe-S cluster 3 in protomer A. Type 7 inhibitors would block the low pH-dependent reduction of Fe-S cluster 2 by reduced Fe-S cluster 4 in protomer A. We have kept the heterodimer portion of the Bakker and Albracht model, since it best explains our results and there is no evidence to suggest that Complex I is not a heterodimer in mammalian mitochondria. Weiss and co-workers have presented convincing evidence [21] that the polypeptide subunits of *Neurospora crassa* mitochondrial Complex I are arranged in an L shaped bipartite structure with one arm embedded in the membrane and the other arm protruding into the matrix. The two arms undergo independent assembly before joining to form a mature functioning Complex I [22]. However, no such structure has yet been observed for mammalian mitochondrial Complex I.

Researchers have long been puzzled at the complexity of mammalian NADH-ubiquinone reductase. No function has yet been ascribed for most of the 42 or more subunits of this electron transport macromolecular complex [1]. If the heterodimer three separate electron pathways model of complex 1 is indeed correct, then perhaps many of the subunits function in modulating and/or facilitating electron transport along the three separate pathways. Numerous EPR studies have failed to delineate the exact pathway of electron flow from NADH to coenzyme Q at pH 8.0, although a some features are fairly well established. Molecular biology methods have been helpful in determining the primary structure of many of the subunits of Complex I [1,23] and perhaps expression systems and site-directed mutagenesis will lead to greater insights into the complexity of this macromolecule. However, the use of specific inhibitors coupled with these other techniques may offer the best chance of success in delineating the complicated patterns of electron flow. Most of the compounds used in this study are highly fluorescent; many are laser dyes. The clever use of photoaffinity analogs of some of these compounds, together with the finding of inhibitors of types 5–7, could be useful in putting together the pieces of this complicated puzzle of electron flow from reduced pyridine nucleotides to coenzyme Q in the mitochondrial respiratory chain.

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