

# Biochemical Studies of *Klebsiella pneumoniae* NifL Reduction Using Reconstituted Partial Anaerobic Respiratory Chains of *Wolinella succinogenes*\*

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In the diazotroph *Klebsiella pneumoniae* the flavoprotein NifL inhibits the activity of the *nif*-specific transcriptional activator NifA in response to molecular oxygen and combined nitrogen. Sequestration of reduced NifL to the cytoplasmic membrane under anaerobic and nitrogen-limited conditions impairs inhibition of cytoplasmic NifA by NifL. To analyze whether NifL is reduced by electrons directly derived from the reduced menaquinone pool, we studied NifL reduction using artificial membrane systems containing purified components of the anaerobic respiratory chain of *Wolinella succinogenes*. In this *in vitro* assay using proteoliposomes containing purified formate dehydrogenase and purified menaquinone (MK<sub>6</sub>) or 8-methylmenaquinone (MMK<sub>6</sub>) from *W. succinogenes*, reduction of purified NifL was achieved by formate oxidation. Furthermore, the respective reduction rates, which were determined using equal amounts of NifL, have been shown to be directly dependent on the concentration of both formate dehydrogenase and menaquinones incorporated into the proteoliposomes, demonstrating a direct electron transfer from menaquinone to NifL. When purified hydrogenase and MK<sub>6</sub> from *W. succinogenes* were inserted into the proteoliposomes, NifL was reduced with nearly the same rate by hydrogen oxidation. In both cases reduced NifL was found to be highly associated to the proteoliposomes, which is in accordance with our previous findings *in vivo*. On the bases of these experiments, we propose that the redox state of the menaquinone pool is the redox signal for *nif* regulation in *K. pneumoniae* by directly transferring electrons onto NifL under anaerobic conditions.

In the free living nitrogen fixing *Klebsiella pneumoniae* transcription of the nitrogen fixation (*nif*) genes is strictly regulated in response to external oxygen and combined nitrogen. Under nitrogen- and oxygen-limiting conditions, transcription of *nif* genes is mediated by the activator protein NifA in combination with the alternative  $\sigma^{54}$ -RNA polymerase (1–3). However, in the presence of molecular oxygen and/or ammonium NifA,

transcriptional activity is inhibited by a second regulatory protein, NifL (4–7). This inhibition of NifA activity by NifL occurs via direct protein-protein interaction in *K. pneumoniae*, which was first implied by co-immunoprecipitation studies (8) and has been recently demonstrated by pulldown experiments (9). A direct protein interaction between NifL and NifA is further consistent with yeast two-hybrid studies and *in vitro* analysis of complex formation between NifL and NifA from *Azotobacter vinelandii*, which also regulates its *nif* gene expression by the two regulatory proteins (10–14).

The negative regulator NifL, which modulates NifA activity in response to the oxygen and nitrogen status of the cell, is a flavoprotein (Refs. 5, 7, 15, and 16; reviewed in Refs. 17–20). By analyzing *in vivo* complex formation between NifA and NifL, we have recently demonstrated that sequestration of the inhibitor NifL to the cytoplasmic membrane under nitrogen and simultaneous oxygen limitation (derepressing conditions) is the key mechanism for regulating cytoplasmic NifA activity in *K. pneumoniae* by NifL (9, 21) (see Fig. 1). We further showed that NifL sequestration to the cytoplasmic membrane is directly dependent on the reduction of its FAD cofactor under anaerobic and nitrogen limitation conditions (9, 21, 22).

The cellular nitrogen status is transduced toward the NifL·NifA regulatory system by direct protein interaction with the nitrogen sensory protein GlnK in both *K. pneumoniae* and *A. vinelandii* (9, 13, 23–27). In *A. vinelandii*, unmodified GlnK activates the inhibitory function of NifL under nitrogen excess by direct protein interaction (28, 29), whereas under nitrogen limitation, the inhibitory activity of *A. vinelandii* NifL appears to be relieved by elevated levels of 2-oxoglutarate (13, 27–32). In contrast to *A. vinelandii*, the relief of NifL inhibition in *K. pneumoniae* under nitrogen limitation depends on the interaction with modified GlnK (23–26). We have recently shown that GlnK effects the cellular localization of NifL in response to nitrogen limitation by direct protein interaction with the inhibitory NifL·NifA complex, resulting in complex dissociation (9) followed by NifL sequestration to the cytoplasmic membrane when oxygen is simultaneously limiting (21) (see Fig. 1).

Recent genetic studies strongly suggest that the electrons, which are responsible for FAD reduction, are derived from the anaerobic respiratory chain of *K. pneumoniae* via the reduced quinone pool (22, 33). To obtain further evidence, we established an *in vitro* system to reduce purified NifL using proteoliposomes containing purified components of the anaerobic

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## NifL Reduction Using an Artificial Membrane System

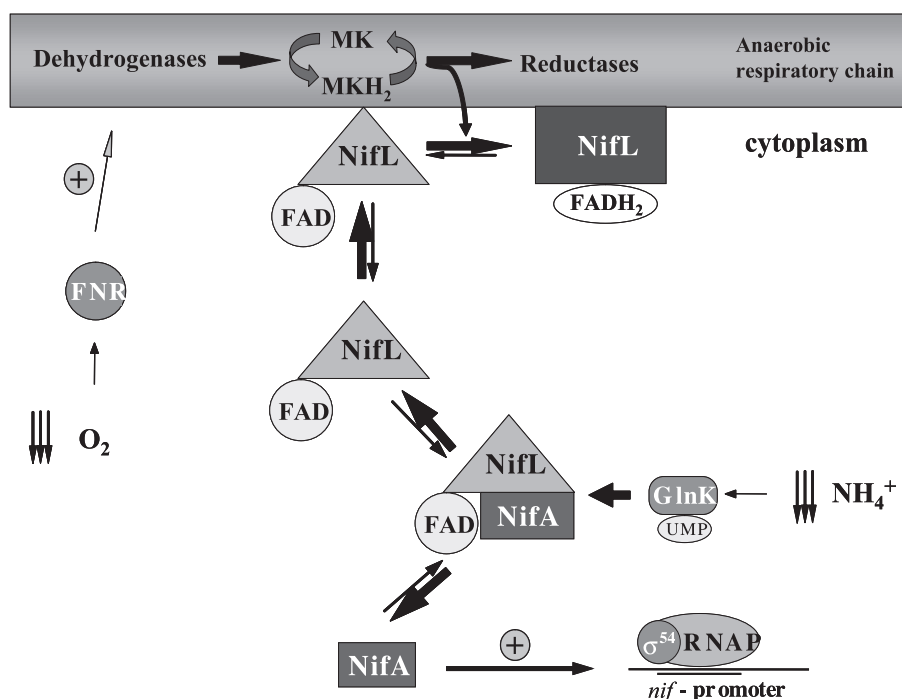


FIGURE 1. **Working model for oxygen and nitrogen signal transduction in *K. pneumoniae*.** Sequestration of cytoplasmic NifL to the cytoplasmic membrane under simultaneous nitrogen and oxygen limiting conditions is the key mechanism of regulating cytoplasmic NifA activity. Interaction with modified GlnK in response to nitrogen limitation results in complex dissociation of the inhibitory NifL-NifA complex (9) followed by NifL reduction at the cytoplasmic membrane by electrons derived from the reduced menaquinone pool (22, 33). *FNR*, fumarate/nitrate reductase regulator; *RNAP*, RNA polymerase.

respiratory chain of the anaerobic rumen bacterium *Wolinella succinogenes* (formate dehydrogenase, hydrogenase, and menaquinones) (34). Studying NifL reduction using this artificial membrane system and the respective electron donors (formate, hydrogen), we achieved strong evidence (i) that under anaerobic conditions electrons are directly transferred from the reduced menaquinone pool in the membrane onto NifL-bound FAD and (ii) that the requirement for any further *K. pneumoniae*-specific protein potentially interacting with NifL, such as a specific oxidoreductase or a NifL-specific receptor protein in the cytoplasmic membrane, can be excluded.

### EXPERIMENTAL PROCEDURES

**Bacterial Strains**—The bacterial strain used in this work was *K. pneumoniae* M5al (wild type) containing pJES794 (35), which carries *malE-nifL* under the control of the *tac* promoter.

**Purification and Reconstitution of the FAD Cofactor of MBP-NifL**—MBP-NifL was synthesized at 30 °C in maximal induction medium (36) in *K. pneumoniae* carrying pJES794 (35). Expression of fusion protein was induced from the *tac* promoter for 4 h with 100  $\mu$ M isopropyl- $\beta$ -D-thiogalactopyranoside at 19 °C when cultures reached a turbidity of  $A_{600} = 0.6$ . After disruption of cells in breakage (B) buffer and centrifugation at 20,000  $\times$  g, fusion proteins were purified from the supernatant by amylose affinity chromatography (16). To reconstitute the fraction of apoflavoprotein, the method was modified in a way that a solution of 10 mM FAD in B-buffer was added to matrix-immobilized MBP-NifL and circulated over the column for 15 h at 4 °C (Fig. 2A) before the elution of MBP-NifL.

To determine the molar extinction coefficient of NifL-bound FAD, at least three independent MBP-NifL purifications were analyzed. Initially the redox spectra of these samples were monitored, and the differences of absorbance at 452 nm were determined ( $\Delta E_{452-600 \text{ nm}}$ ) for each sample. Subsequently, the FAD cofactor of the respective MBP-NifL fractions was extracted with trichloroacetic acid as recently described (16), and the amounts of extracted FAD were calculated by fluorescent quantification (excitation at 465 nm and monitoring emission at 520 nm) using FAD solutions in B-buffer containing 5% trichloroacetic acid as standards. The absorbance difference ( $\Delta E_{452-600 \text{ nm}}$ ) obtained from the redox spectra and the respective amount of extracted FAD were used to calculate the molar extinction coefficient that was determined to be  $\epsilon_{452-600 \text{ nm}} = 6.63 \text{ mM}^{-1} \times \text{cm}^{-1}$ . Using this molar extinction coefficient, the FAD content of purified MBP-NifL fractions was

routinely calculated from their respective redox spectra (Fig. 2B). Reconstituted MBP-NifL contained between 0.9 and 1.0 mol of FAD cofactor per mol of MBP-NifL. In general, only fully reconstituted MBP-NifL protein charges (1 mol FAD/mol MBP-NifL) were used in the following reduction assays.

**Purification of Formate Dehydrogenase, Hydrogenase, and Menaquinones of *W. succinogenes***—Formate dehydrogenase and hydrogenase of *W. succinogenes* were isolated as described previously (37–39). The quinones of *W. succinogenes* menaquinone<sub>6</sub> (MK<sub>6</sub>)<sup>2</sup> and 8-methylmenaquinone<sub>6</sub> (MMK<sub>6</sub>) were extracted from the membrane fraction using a mixture of petrol ether and methanol (70/30 v/v). The different quinones in the extract were separated by high performance liquid chromatography (HPLC) according to Unden (40). The separated quinones were quantified by HPLC using vitamin K<sub>1</sub> (Fluka) as a standard (34).

**Preparation of Proteoliposomes**—Sonic liposomes containing menaquinones from *W. succinogenes* were prepared from a mixture of egg phospholipids (95%, w/w) (41) and phosphatidylethanolamine (5%, Fluka no. 60650) as described (42). Proteoliposomes were prepared by freeze-thawing sonic liposomes containing the quinones indicated and either hydrogenase or formate dehydrogenase as described by Unden and Kröger (37).

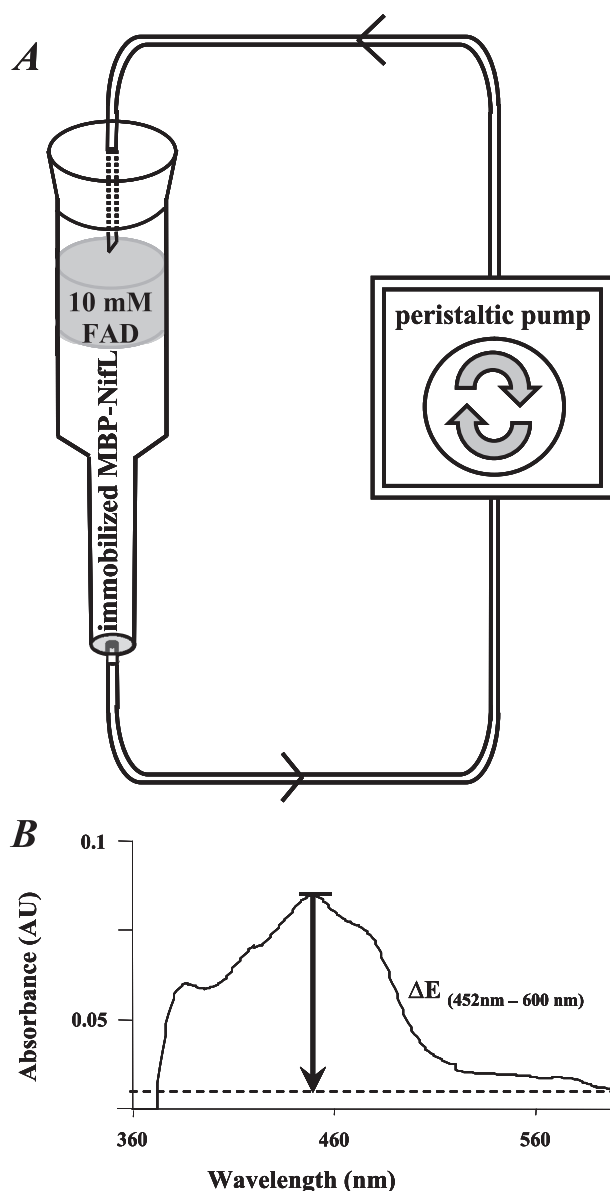
<sup>2</sup> The abbreviations used are: MK<sub>6</sub>, menaquinone<sub>6</sub>; DMN, dimethylnaphtoquinone; MMK<sub>6</sub>, 8-methylmenaquinone<sub>6</sub>; PutA, proline utilization protein A; Fdh, formate dehydrogenase.

*Reduction of MBP-NifL by Formate Oxidation or Hydrogen Oxidation Using Artificial Membrane Systems*—All analyses of MBP-NifL reduction were performed in the absence of oxygen under strictly anaerobic conditions in volume-reduced glass cuvettes. The reduction of MBP-NifL by formate (or hydrogen) was recorded as the absorbance difference at 452–600 nm ( $\Delta\epsilon_{452-600\text{ nm}} = 6.63\text{ mM}^{-1} \times \text{cm}^{-1}$ ) in B-buffer containing a constant amount of 4.2 nmol of MBP-NifL in a total test volume of 400  $\mu\text{l}$  and solubilized formate dehydrogenase (or hydrogenase, respectively) or in proteoliposomal membranes (5 mg of phospholipid in a 400- $\mu\text{l}$  test volume) and integrated formate dehydrogenase with amounts indicated in Table 1. The additional electron mediator dimethylnaphthoquinone (DMN) or MK in proteoliposomal membranes was also added as indicated in Table 1. The redox reaction was started by the addition of an initial concentration of 12.5 mM formate (or by the addition of molecular hydrogen). The reduction of MBP-NifL was archived by recording spectra from 600 to 360 nm in timed intervals using a spectral photometer (Spektrophotometer U-3000, Hitachi). The difference in absorbance at 452 nm, corrected for the difference in absorbance at 600 nm (turbidity and additives) of the resulting redox spectra, was used to calculate the amount of MBP-NifL reduction rate by time using the extinction coefficient of MBP-NifL bound FAD determined earlier ( $\Delta\epsilon_{452-600\text{ nm}} = 6.63\text{ mM}^{-1} \times \text{cm}^{-1}$ ).

*Analyzing Membrane Association of Reduced MBP-NifL by Sucrose Gradient Centrifugation*—In a test volume of 250  $\mu\text{l}$ , 6.1 nmol of MBP-NifL were fully reduced by an excess of formate (15 mM) using proteoliposomes (6.25 mg of phospholipids) containing purified formate dehydrogenase (2.5 nmol, *i.e.* 64  $\mu\text{g}/\text{mg}$  phospholipid) and menaquinone<sub>6</sub> (18 nmol/mg phospholipid) from *W. succinogenes* (34). The complete assay was subsequently subjected under anaerobic conditions to an anaerobic sucrose gradient (10–40% sucrose in B-buffer complemented with 15 mM formate by a total volume of 28 ml). After ultracentrifugation at  $175,000 \times g$  for 270 min formate dehydrogenase activity of the different fractions was assayed as described recently (43), and the various fractions were analyzed for NifL presence by Western blot analysis. For ultracentrifugation experiments in the presence of oxygen, the presence of formate dehydrogenase in the fractions was analyzed by Western blot analysis. Fractions were separated on denaturing polyacrylamide gels and transferred to nitrocellulose membranes (BioTrace<sup>®</sup>NT, Pall Life Science) (44). Membranes were subsequently exposed to specific polyclonal rabbit antisera directed against MBP-NifL of *K. pneumoniae* or formate dehydrogenase of *W. succinogenes*. Protein bands were detected with secondary antibodies directed against rabbit immunoglobulin G and coupled to horseradish peroxidase (Bio-Rad), visualized, and quantified using the ECLplus system (Amersham Biosciences) with a fluorimager (DianaIII, Raytest).

## RESULTS

The goal of this work was to gain a deeper insight into the NifL reduction in *K. pneumoniae* resulting in membrane sequestration of the negative regulator under simultaneous nitrogen and oxygen limiting conditions. To address the question of whether electrons are directly transferred from the



**FIGURE 2. Reconstitution of immobilized MBP-NifL with FAD.** MBP-NifL was expressed in *K. pneumoniae* and purified as described under “Experimental Procedures.” **A**, MBP-NifL of the cell extracts was immobilized to the affinity chromatography matrix; subsequently a 10 mM FAD solution was added and circulated over the column for 15 h at 4 °C, yielding in a 100% reconstitution of the FAD-cofactor of MBP-NifL. **B**, UV-visible difference spectrum (oxidized-reduced) of fully reconstituted MBP-NifL (9.1  $\mu\text{M}$ ), oxidized with molecular oxygen, and reduced with 5 mM dithionite.

reduced quinone pool onto the flavoprotein NifL or via a NifL-specific protein (reductase or receptor), we studied NifL reduction using a partial reconstituted anaerobic respiratory chain from *W. succinogenes*.

*Purification of Fully Reconstituted NifL Protein and Components of the W. succinogenes Anaerobic Respiratory Chain*—After MBP-NifL synthesis in *K. pneumoniae* under low temperatures (19 °C), reconstitution of the FAD cofactor into the flavoprotein was achieved by continuous circulation of a 10 mM FAD solution over matrix-immobilized MBP-NifL at 4 °C (see Fig. 2 and “Experimental Procedures”). The resulting purified MBP-NifL fractions, which in general contained approximately 1 mol of FAD-cofactor/mol of MBP-NifL, were used in the *in*

## NifL Reduction Using an Artificial Membrane System

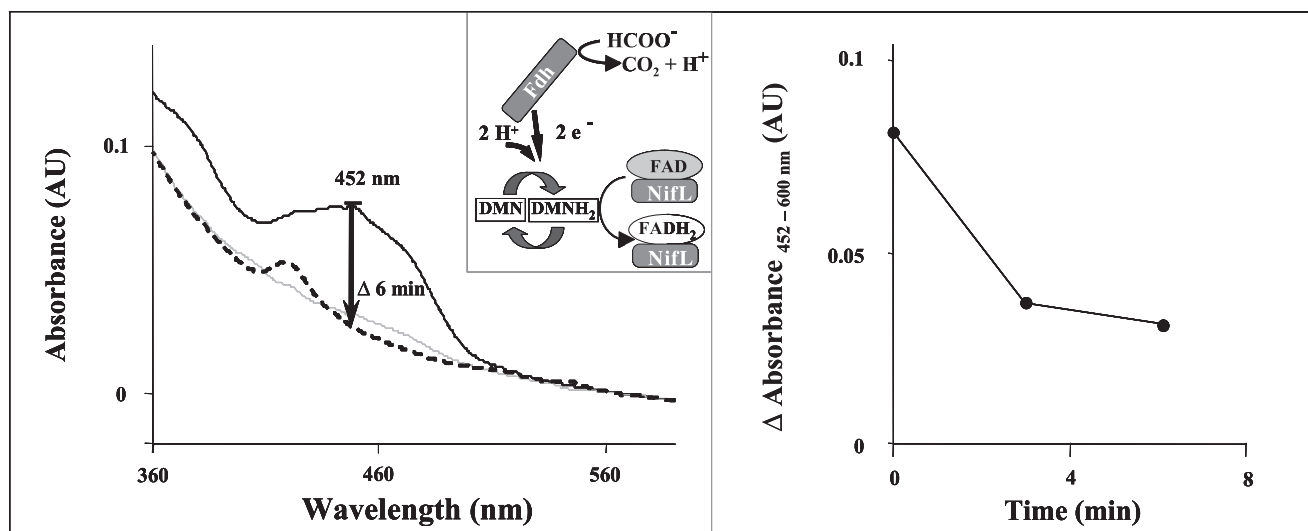


FIGURE 3. MBP-NifL reduction by formate in the presence of solubilized membranous formate dehydrogenase of *W. succinogenes* and DMN. Electrons derived from formate oxidation were transferred to the FAD cofactor of purified MBP-NifL (4.2 nmol) in the presence of solubilized formate dehydrogenase (Fdh) isolated from *W. succinogenes* and in the presence of 0.2 mM water-soluble quinone analog DMN as the electron mediator. UV-visible spectra were monitored every 2 or 3 min as described under "Experimental Procedures." After 6 min, reduction of  $\sim 3.0$  nmol of MBP-NifL was achieved. Left panel: solid bold line, original data of the initial spectrum; solid fine line, after 3 min; dashed line, after 6 min. The right panel shows the change in absorbance at 452 nm corrected for the absorbance at 600 nm versus incubation time. The electron flow in the *in vitro* system from formate to MBP-NifL is depicted in the schematic.

*in vitro* reduction assays described below. The respective molar extinction coefficient at 452 nm for NifL-bound FAD was determined to be  $\Delta\epsilon_{452-600\text{ nm}} = 6.63\text{ mM}^{-1} \times \text{cm}^{-1}$  as described under "Experimental Procedures." For the reconstitution of partial anaerobic respiratory chains in liposomes, native formate dehydrogenase, native hydrogenase, and both menaquinones (MK<sub>6</sub> and MMK<sub>6</sub>) were purified from membranes of *W. succinogenes* as described by Dietrich and Klimmek (34) and Biel *et al.* (43).

**Reduction of MBP-NifL by Formate Oxidation Catalyzed by Solubilized Membranous Formate Dehydrogenase of *W. succinogenes***—All the following analyses of MBP-NifL reduction were performed in the absence of oxygen under strictly anaerobic conditions in a test volume of 400  $\mu\text{l}$  (see "Experimental Procedures"). As an initial control, formate oxidation by purified solubilized formate dehydrogenase from membranes of the anaerobic rumen bacterium *W. succinogenes* was studied, which demonstrated that cytochrome *b* of *W. succinogenes* formate dehydrogenase (34  $\mu\text{g}$ ) was fully reduced in less than 1 min after adding 12.5 mM formate (data not shown). Thus, in the following test assays 12.5 mM formate was routinely added as electron donor. To analyze whether electrons derived from formate oxidation can in principle be transferred to and reduce MBP-NifL in solution, formate was oxidized by purified solubilized *W. succinogenes* formate dehydrogenase (34  $\mu\text{g}$ ) in the presence of 4.2 nmol of purified MBP-NifL in the test assay. In the presence of 0.2 mM of the water-soluble quinone DMN, which is reduced by the cytochrome *b* subunit of the formate dehydrogenase by formate and which acts as an electron mediator, MBP-NifL was significantly reduced in this *in vitro* assay. The kinetics of the respective reduction of NifL-bound FAD cofactor was monitored by detecting the absorbance between 650 and 360 nm every 2 min as described under "Experimental Procedures." As shown in Fig. 3,  $\sim 3.0$  nmol of MBP-NifL were reduced within 6 min after starting the reaction by the addition

of 12.5 mM formate to the assay. In the absence of formate no MBP-NifL reduction was observed. In the absence of DMN but in the presence of formate MBP-NifL reduction occurred about 2 orders of magnitude slower than with the presence of the redox mediator DMN (see Table 1, Experiment 3). To calculate the amount of FAD reduction of MBP-NifL by formate oxidation, the absorbance decrease by time at 452 nm and the extinction coefficient for MBP-NifL-bound FAD ( $\Delta\epsilon_{452-600\text{ nm}} = 6.63\text{ mM}^{-1} \times \text{cm}^{-1}$ ) determined earlier was used. The UV-visible spectra in Fig. 3 further indicate that electrons derived from formate oxidation by formate dehydrogenase are subsequently transferred to DMN and later to the NifL-bound FAD, as no reduced cytochrome *b* of formate dehydrogenase is visible (Fig. 3, absorbance at 424 and 552 nm).

**MBP-NifL Reduction Driven by Proteoliposomes Containing Partial Reconstituted Anaerobic Respiratory Chains of *W. succinogenes***—The next question we addressed was to analyze whether MBP-NifL can be reduced by electrons derived from reconstituted anaerobic respiratory chains. To test whether an electron transfer from membrane-integrated reduced menaquinones to MBP-NifL can be achieved, proteoliposomes were prepared by inserting purified formate dehydrogenase or hydrogenase and menaquinones (MK<sub>6</sub> or MMK<sub>6</sub>), isolated from membranes of *W. succinogenes* into liposomes as described under "Experimental Procedures." The components of the respiratory chain and their relative amounts were varied to further characterize the potential reduction and the rate of MBP-NifL reduction by electrons derived from the reconstituted anaerobic respiratory chains. The reduction of 4.2 nmol MBP-NifL generally used in the 400- $\mu\text{l}$  test assays was studied under the respective conditions by monitoring the UV-visible spectra every 2 min after starting the reaction by the addition of 12.5 mM formate (final concentration).

Unexpectedly, in control assays using proteoliposomes, which solely contained purified formate dehydrogenase (27.2

TABLE 1

Characterization of *in vitro* reduction of 4.2 nmol MBP-NifL (400- $\mu$ l test volume) by formate oxidation using formate dehydrogenase (Fdh) from *W. succinogenes*

Experiment	Solubilized Fdh amount	DMN amount	Initial FAD reduction $\Delta E_{\text{initial}}^a$	Reduced FAD	$\Delta$ Time	Figure depicted
	$\mu\text{g}$		$\text{min}^{-1}$	$\text{nmol}$	$\text{min}$	
3	34	None	0.00076	2.7	>45.0	Not shown
2	34	0.2 mM	0.015	2.92	6.0	Fig. 3
	Fdh amount in proteoliposomes	MK <sub>6</sub> amount	Initial FAD reduction $\Delta E_{\text{initial}}^a$	Reduced FAD	$\Delta$ Time	Figure depicted
	$\mu\text{g}/\text{mg phospholipid}^{-1}$	$\text{mg phospholipid}^{-1}$	$\text{min}^{-1}$	$\text{nmol}$	$\text{min}$	
5	13.6	0.01 $\mu\text{mol}$	0.0036; $1 \times^b$	2.8	21.0	Not shown
6	27.2	0.01 $\mu\text{mol}$	0.014; $4 \times^b$	3.11	8.0	Fig. 5A
11	27.2	0.026 $\mu\text{mol}$	0.032; $9 \times^b$	3.32	4.3	Fig. 5B
12	13.6	0.026 $\mu\text{mol}$	0.0124; $4 \times^b$	2.84	12.0	Not shown
9	27.2	None	0.002; $0.14 \times^b$	3.44	21.0	Fig. 4
10	13.6	None	0.001; $0.07 \times^b$	2.16	77.0	Not shown
13	27.2	0.017 $\mu\text{mol MMK}_6$	0.015; $4 \times^b$	1.76	8.0	Not shown

<sup>a</sup> The reduction rates were calculated by determining the difference of the flavin-specific absorbance 2 min after formate addition.

<sup>b</sup> The reduction rates under the different conditions are indicated in relation to the reduction rate obtained under conditions present in Experiment 5, which was set to 1.0.

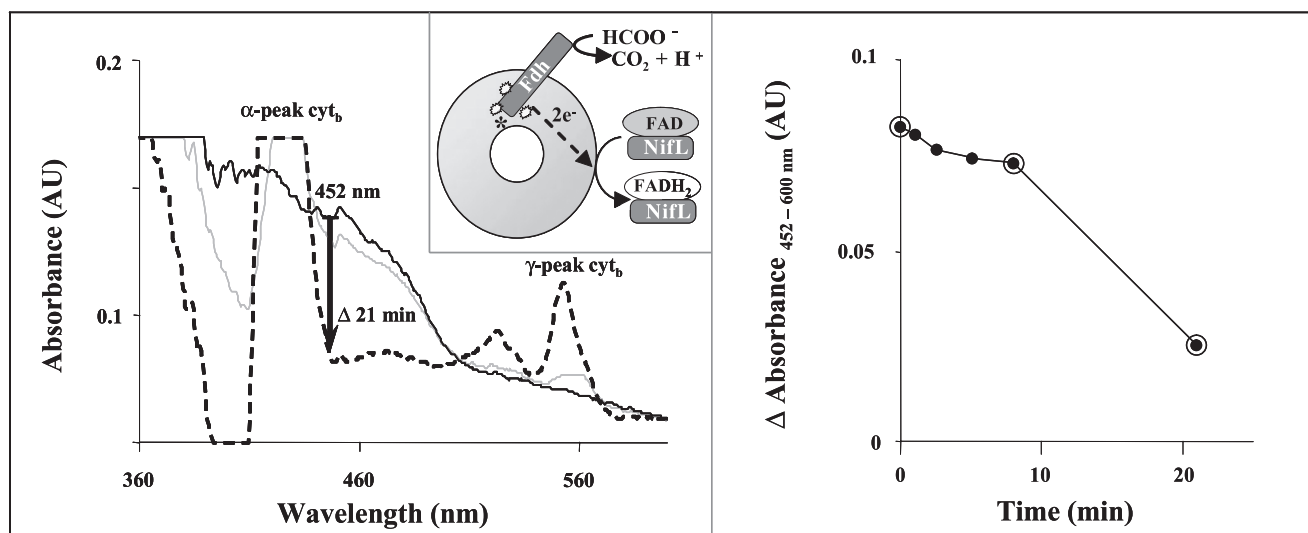


FIGURE 4. Reduction of MBP-NifL by formate oxidation using proteoliposomes containing purified formate dehydrogenase from *W. succinogenes* in the absence of any additional electron carrier. The FAD cofactor of purified MBP-NifL (4.2 nmol) was reduced by electrons derived from formate oxidation by purified *W. succinogenes* Fdh reconstituted into sonic liposomes (27.2  $\mu\text{g}$  of formate dehydrogenase/mg of phospholipid). The UV-visible spectra were monitored every 3–10 min. After 21 min 3.44 nmol of MBP-NifL was reduced by electrons derived from formate oxidation. This is due to the presence of contaminating menaquinones (\*) present in the purified formate dehydrogenase protein fraction (0.1–0.2 mol of MK  $\times$  mol of formate dehydrogenase<sup>-1</sup>), which apparently allowed an electron transfer to NifL as indicated in the schematic (dashed line). Left panel: bold dark line, original data of the initial spectrum; fine gray line, after 8 min; dashed bold line, after 21 min. The absorbance at 424 nm ( $\alpha$ -peak) and 562 nm ( $\gamma$  peak) is due to the reduction of formate dehydrogenase cytochrome *b*, which occurs during the reduction process, indicating that the small amount of the menaquinone in the liposomal membrane is fully reduced. The right panel shows the change in absorbance at 452 nm corrected for the absorbance at 600 nm versus incubation time. The respective time points, for which the corresponding spectra are shown, are indicated by additional circles. The electron flow in the *in vitro* system from formate to MBP-NifL is depicted in the schematic.

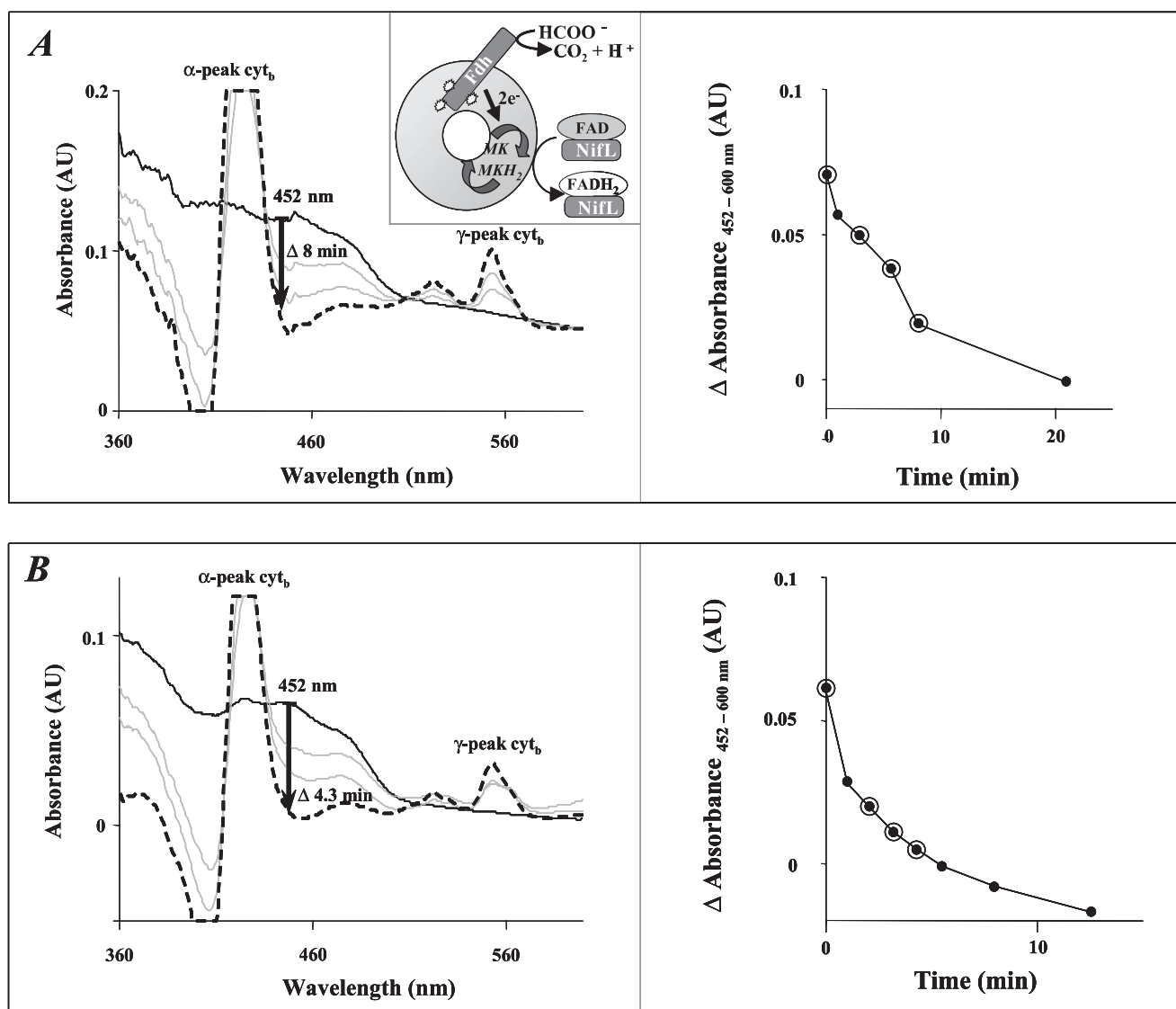
$\mu\text{g}/\text{mg}$  phospholipids) in the absence of any additional menaquinones as electron carriers, significant reduction of MBP-NifL (3.44 nmol) was detected within a long time of 21 min as demonstrated in Fig. 4. This finding strongly indicates that electrons derived from formate oxidation have been finally transferred onto MBP-NifL. As seen from the spectrum (Fig. 4, absorbance at 424 and 562 nm), cytochrome *b* of the formate dehydrogenase was reduced within less than 1 min. It appears that the contaminating menaquinones, which are present in very small amounts in the fraction of purified formate dehydrogenase (0.1–0.2 mol of MK  $\times$  mol of formate dehydrogenase<sup>-1</sup>),<sup>3</sup> allowed the electron transfer from reduced cytochrome *b* of formate dehydrogenase onto NifL by slow rates (Fig. 4, left panel, inset,

dashed line). This finding is further supported by the fact that isolated Triton-X100-solubilized formate dehydrogenase was also able to reduce MBP-NifL in the absence of the additional water-soluble electron mediator DMN; however, a reaction time longer than 45 min was required to reduce 2.7 nmol of MBP-NifL in the presence of formate and formate dehydrogenase (Table 1, Experiment 3).

When proteoliposomes were used in the *in vitro* assay, which contained in addition to formate dehydrogenase (27  $\mu\text{g}/\text{mg}$  phospholipids) MK<sub>6</sub> isolated from membranes of *W. succinogenes* (0.01  $\mu\text{mol}$  MK<sub>6</sub>/mg phospholipid), MBP-NifL reduction was achieved significantly faster than in the absence of any added quinones (Table 1, Experiment 6). As shown in Fig. 5A, 3.1 nmol of MBP-NifL was reduced within 8 min after starting by the addition of formate. This finding indicates that the elec-

<sup>3</sup> O. Klimmek, unpublished results.

## NifL Reduction Using an Artificial Membrane System



**FIGURE 5. MBP-NifL reduction by formate oxidation using proteoliposomes containing purified formate dehydrogenase and MK<sub>6</sub> from *W. succinogenes*.** The NifL-bound FAD cofactor (4.2 nmol) was reduced by formate oxidation using proteoliposomes containing purified Fdh (27 μg of formate dehydrogenase/mg of phospholipid) and purified MK<sub>6</sub> from *W. succinogenes*. MBP-NifL reduction was analyzed by monitoring the UV-visible spectra every 2 min. 3.11 nmol of MBP-NifL were reduced within 8 min when using 0.01 μmol of MK<sub>6</sub>/mg of phospholipid (A) and 3.32 nmol within 4.3 min using 0.026 μmol of MK<sub>6</sub>/mg of phospholipid (B). In the left panels the original data are depicted. **Bold dark lines**, the initial spectra; **solid fine lines**, after 2.75 and 5.7 min (A) and after 2 and 3.15 min (B); **bold dashed line**, after 8 min (A) and after 4.3 min (B). The absorbance at 424 and 562 nm represents the reduction of Fdh cytochrome *b*. The right panels show the change in absorbance at 452 nm corrected for the absorbance at 600 nm versus incubation time; the respective time points, for which the corresponding spectra are shown, are indicated by additional circles. The electron flow in the *in vitro* system from formate to MBP-NifL is depicted in the schematic.

trons from the reduced formate dehydrogenase cytochrome *b* are transferred onto MBP-NifL in a much more efficient way via the additional menaquinones as electron carriers (Fig. 5A, left panel, inset). The reduction rate was even further increased when using higher amounts of menaquinones inserted into the liposomes (0.026 μmol of MK<sub>6</sub>/mg of phospholipid) by a constant formate dehydrogenase content; reduction of 3.32 nmol of MBP-NifL was achieved within 4.3 min (see Fig. 5B). These findings strongly indicate that the reduction of MBP-NifL is directly dependent on the menaquinone concentration in the membrane of the proteoliposomes. The reduction is further directly dependent on the formate dehydrogenase concentration in the proteoliposomes, as reducing the amount of formate dehydrogenase inserted in proteoliposomes to 13.6 μg/mg of

phospholipid resulted in a significant increase of reaction time for NifL reduction (Table 1, compare Experiment 5 with Experiment 6). Besides MK<sub>6</sub>, also MMK<sub>6</sub>, which comprises a much deeper redox potential (−220 mV),<sup>4</sup> inserted into proteoliposomes together with formate dehydrogenase allowed the reduction of MBP-NifL in the *in vitro* assay with a comparable rate (see Table 1, compare Experiments 13 and 6). The overall finding that electrons from reduced menaquinones of reconstituted partial anaerobic respiratory chains can be directly transferred onto MBP-NifL strongly supports our hypothesis that under oxygen-limiting conditions NifL is able to receive elec-

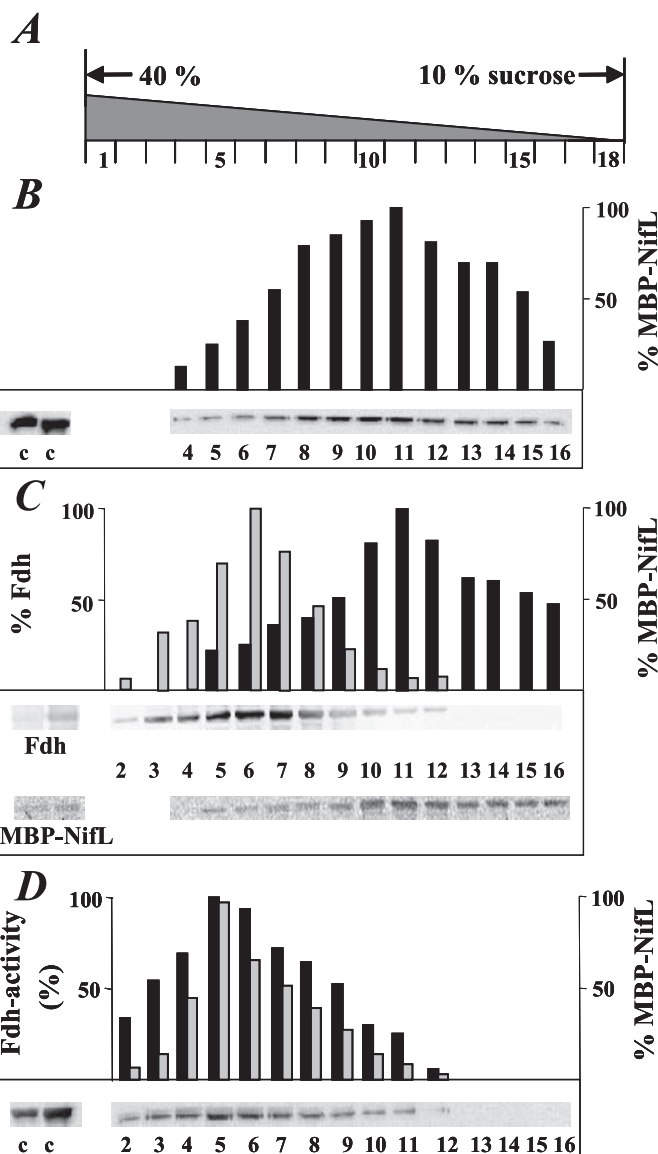
<sup>4</sup> O. Klimmek and F. MacMillan, unpublished results.

trons from the reduced menaquinone pool of the anaerobic respiratory chain in *K. pneumoniae*.

To compare the reduction rates for MBP-NifL in the *in vitro* assays using different amounts of formate dehydrogenase and MK<sub>6</sub> inserted into the proteoliposomes, we determined the initial NifL reduction rates under the respective conditions. The initial reduction rates were calculated by determining the difference of the flavin-specific absorbance at 452 nm corrected for the absorbance at 600 nm 2 min after formate addition. The rates obtained, which are summarized in Table 1 (initial FAD reduction), clearly demonstrate that the rate of NifL reduction is directly dependent on the formate dehydrogenase content and the menaquinone concentration in the proteoliposomal membrane. In general, an approximate 4-fold increase of the reduction rate was achieved by a 2-fold increase of the menaquinone concentration in the liposomal membrane (Table 1, compare Experiments 5 and 12) or the formate dehydrogenase content in the proteoliposomes used (Table 1, compare Experiments 5 and 6). Increasing both components in the membrane simultaneously resulted in a 9-fold increase of the reduction rate of MBP-NifL (Table 1, compare Experiments 5 and 11). Comparing the reduction rates of MBP-NifL when using proteoliposomes in the absence and presence of additional menaquinones also clearly showed that the contaminating MK of purified formate dehydrogenase are not sufficient for high reduction rates and that a 7-fold increase in electron transfer rates is achieved in the presence of additional MK<sub>6</sub> (Table 1, compare Experiments 6 and 9).

As an independent control to confirm the data obtained, purified *W. succinogenes* hydrogenase and MK<sub>6</sub> were inserted into liposomes. Using those proteoliposomes, MBP-NifL reduction was achieved by hydrogen oxidation with comparable reduction rates as the ones determined with formate dehydrogenase (data not shown).

**Association of Reduced MBP-NifL to Proteoliposomal Membranes**—Using two independent approaches, we have recently shown *in vivo* (i) that under derepressing conditions NifL is membrane-associated, allowing cytoplasmic NifA to activate *nif*-gene expression and (ii) that the membrane sequestration is directly dependent on FAD reduction (9, 21, 22). To confirm our previous results, we further analyzed whether MBP-NifL, which was reduced in the *in vitro* assay by electrons derived from formate oxidation by a reconstituted partial anaerobic respiratory chain, is associated with the proteoliposomes or not. To study potential association of reduced MBP-NifL to proteoliposomal membranes, we reduced 6.1 nmol of MBP-NifL with 15 mM formate using proteoliposomes (6.25 mg of phospholipids) containing formate dehydrogenase and MK<sub>6</sub> (64 μg of formate dehydrogenase and 18 nmol of MK<sub>6</sub> per mg of phospholipid). After 20 min of anaerobic incubation, the complete assay was applied to a 28-ml sucrose gradient (10–40% sucrose in B-buffer) containing 15 mM formate to keep MBP-NifL constantly in a reduced state. After ultracentrifugation for 4.5 h performed under anaerobic conditions, the different fractions (1.5 ml) were analyzed for (i) the presence of MBP-NifL by Western blot analysis using polyclonal antibodies raised against NifL and (ii) the presence of formate dehydrogenase activity as described under “Experimental Procedures” to detect fractions



**FIGURE 6. Analysis of association of *in vitro* reduced MBP-NifL to proteoliposomal membranes.** MBP-NifL (610 μg) was loaded onto a 28-ml sucrose gradient (10–40% sucrose in B-buffer) followed by ultracentrifugation for 4.5 h at 175,000 × *g*. The respective fractions of the gradient depicted in A were analyzed for the presence of MBP-NifL by Western blot analysis using antibodies directed against NifL. In general, fractions of one experiment were analyzed on the same polyacrylamide gel together with purified MBP-NifL as control. B, ultracentrifugation of MBP-NifL under aerobic conditions in the absence of proteoliposomes. Shown are original Western blot data (bottom) and relative amounts of NifL in the respective fractions setting the NifL amount of fraction 11 to 100% (black bars); c, 210 and 260 ng of purified MBP-NifL. C, ultracentrifugation of MBP-NifL under aerobic conditions in the presence of proteoliposomes. Shown are original Western blot data (bottom) and relative amounts of NifL and Fdh in the respective fractions setting the NifL amount of fraction 11 to 100% (black bars) and the Fdh amount of fraction 6 to 100% (gray bars). D, anaerobic ultracentrifugation of MBP-NifL after *in vitro* reduction by proteoliposomes (see “Experimental Procedures”). The relative amounts of NifL, setting the NifL amount of fraction 5 to 100%, are indicated as black bars above the original Western blot data. The Fdh activity of the respective fractions was determined as described under “Experimental Procedures.” The relative amounts are indicated as gray bars, setting the Fdh activity of fraction 5 (97 units/ml) to 100%.

containing proteoliposomes with formate dehydrogenase. The results, which are depicted in Fig. 6, clearly demonstrated the simultaneous presence of reduced MBP-NifL and formate dehydrogenase in proteoliposomes mainly in fractions 4–8

## NifL Reduction Using an Artificial Membrane System

(Fig. 6D). However, when oxidized MBP-NifL and proteoliposomes were analyzed in the presence of oxygen, the majority of MBP-NifL was found in fractions 9–16 (Fig. 6C) as was the case for oxidized MBP-NifL in the absence of proteoliposomes (Fig. 6B). The finding that exclusively reduced MBP-NifL is associated to the proteoliposomes strongly confirms our previous *in vivo* results that NifL is membrane associated under derepressing conditions. It further clearly demonstrates that no additional *K. pneumoniae*-specific protein is required for NifL reduction or membrane association of reduced NifL.

### DISCUSSION

The enzymatic reduction of molecular nitrogen to ammonia is an extremely energy consuming process, and the presence of oxygen causes the catalyzing nitrogenase complex to be rapidly and irreversibly inactivated (45, 46). As a consequence diazotrophs tightly control both the synthesis and activity of nitrogenase in response to environmental changes in oxygen and ammonium availability. In *K. pneumoniae*, *nif* genes are exclusively expressed under oxygen and concurrent nitrogen limitation (for review, see Refs. 17 and 18). Here the key regulatory mechanism for *nif* gene induction by the transcriptional activator NifA is membrane sequestration of the reduced negative regulator NifL under simultaneous oxygen and nitrogen limitation (9, 21, 22). The focus of this work was to investigate the proposed electron transfer from the anaerobic respiratory chain onto NifL, resulting in membrane association of NifL.

*Demonstration of a Direct Electron Transfer from Reduced Menaquinones of Artificial Membrane Systems to NifL*—Using reconstituted partial anaerobic respiratory chains of *W. succinogenes* as artificial membrane systems consisting of defined components, we revealed strong evidence that electrons are directly transferred from reduced menaquinone pools in membranes to the FAD cofactor of soluble NifL (Figs. 4 and 5). This is consistent with previous experiments in which NifL reduction has been achieved by the anaerobic respiratory chain of inside-out vesicles derived from anaerobic *K. pneumoniae* cells after the addition of NADH/H<sup>+</sup> (22). The current experimental data clearly demonstrate for the first time that NifL is able to receive electrons directly from reduced menaquinones in membranes in the absence of any additional oxidoreductase or any *K. pneumoniae*-specific protein. This strongly indicates that neither a NifL-specific membrane-bound receptor is required for NifL contacting the cytoplasmic membrane nor is a *K. pneumoniae* specific electron carrier or oxidoreductase required for the reduction of the NifL-bound FAD cofactor. This is further supported by the results obtained from a saturated transposon mutagenesis, in which despite components of the anaerobic respiratory chains of *K. pneumoniae*, fumarate/nitrate reductase regulator, and essential genes for nitrogen fixation (e.g. *nif* genes, *ntxB*, *ntxC*, *glnK*), no further specific proteins were identified to be essential for NifL reduction.<sup>5</sup>

Sucrose gradient ultracentrifugation analysis further showed that the majority of NifL reduced by the artificial membrane systems stays associated to the proteoliposomes (Fig. 6D). In

the presence of oxygen, however, oxidized NifL was mainly present in fractions with significantly lower sucrose concentration not containing the proteoliposomes (Fig. 6C). This finding that only reduced NifL stays associated to the proteoliposomes *in vitro* is in accordance with our previous *in vivo* results on NifL localization in response to oxygen availability (21) (see Fig. 1). NifL presumably contacts the cytoplasmic membrane with its hydrophobic N-terminal domain based on either ionic or hydrophobic interactions. However, at the current experimental status we are not able to analyze (e.g. by incorporation of protonophores) whether membranes have to be energized or not for NifL contact. This is due to the fact that the oxidoreductases (formate dehydrogenase and hydrogenase) were inserted into the proteoliposomes by the method of freeze and thawing leading to a more or less random orientation of the enzymes in the proteoliposomes and to more or less non-proton dense liposomes that possibly do not generate a membrane potential. For a distinct orientation of the dehydrogenases in the liposomal membranes and for good impermeability of protons, experimentally difficult methods with a high investment of time and work are necessary, e.g. preparation methods of proteoliposomes according to Lambert *et al.* (47).

After changes from aerobic to anaerobic environmental conditions under nitrogen limitation, rapid reduction of NifL is important to sequester the negative regulator to the membrane. The rates of NifL reduction achieved in the *in vitro* system were calculated to be ~2 nmol of NifL/min when the highest amounts of quinones were used in the proteoliposomes with a MK<sub>6</sub> to NifL ratio in the range of 30 to 1 (Experiment 11). Under nitrogen limitation ~100 molecules of NifL are present in a single *K. pneumoniae* cell,<sup>6</sup> whereas the amount of reduced menaquinones in the membranes in the steady state of anaerobic respiration is approximately 3 orders of magnitude higher. This *in vivo* ratio should allow much higher rates of NifL reduction *in vivo* than obtained *in vitro*; consequently, under derepressing conditions, each NifL protein of a cell which contacts the cytoplasmic membrane will immediately receive electrons from the reduced menaquinone pool. Thus, this unspecific electron transfer from reduced menaquinones onto NifL under physiological conditions should be sufficient to sequester NifL to the membrane and lead to a *nif* gene induction in a short time.

A comparable regulatory mechanism has been described for the proline utilization protein A (PutA) in *Escherichia coli* and *Salmonella typhimurium*. The flavoprotein PutA acts as a transcriptional repressor of the proline utilization (*put*) operon by binding to an operator when intracellular proline concentrations are low (48–50). However, high intracellular proline concentrations have been shown to alter the function of PutA driven by the reduction of PutA-bound FAD (51–53). Reduced PutA associates with the membrane and catalyzes the two-step oxidation of proline to glutamate. Membrane sequestration of PutA disrupts the PutA-DNA complex and consequently induces the *put* gene expression (52, 54, 55).

In contrast to *K. pneumoniae* NifL, membrane association has not been demonstrated for the redox-sensitive NifL protein

<sup>5</sup> J. Stips and R. A. Schmitz, unpublished results.

<sup>6</sup> R. Thummer and R. A. Schmitz, unpublished data.



of *A. vinelandii*, nor has the physiological electron donor been identified yet. It is currently proposed that under anaerobic conditions reduction of *A. vinelandii*-NifL occurs non-specifically and is dependent on the availability of reducing equivalents in the cytoplasm (15, 19, 20, 56). Under simultaneous nitrogen limitation, the NifL conformation with a reduced FAD moiety does not allow interaction and inhibition of cytoplasmic NifA. However, upon oxidation of its FAD moiety, NifL undergoes a conformational change and is competent to inhibit NifA irrespectively of the nitrogen status (for review, see Refs. 19 and 20).

**Hypothetical Model: Menaquinones as the Redox Signal for *K. pneumoniae* NifL**—In previous work we hypothesized that the *nif* gene regulation is based on NifL monitoring the reduction state of components of the anaerobic respiratory chain. The presented work now clearly demonstrates a direct electron transfer from reduced menaquinones onto NifL and provides evidence for a direct link of the redox status of the menaquinone pool to *nif* gene regulation. Thus, the reduced menaquinone pool appears to act as the signal for anaerobiosis. To our knowledge only very few examples are known for which the redox state of the quinone pool is proposed as the redox signal for downstream signal transduction to regulate gene expression; (i) the Arc two-component regulatory system of *E. coli* (57), (ii) histidine kinases BvgS and EvgS of *Bordetella pertussis* and *E. coli* (58), and (iii) RegB of *Rhodobacter capsulatus* (59). Georgellis and co-workers (60) showed that in *E. coli*, oxidized quinones act as direct negative signal inhibiting autophosphorylation of ArcB by oxidizing two redox active cysteine residues, leading to intermolecular disulfide bond formation. In the case of the global redox two component regulatory system RegA/RegB of *R. capsulatus*, Bauer and co-workers (59) very recently demonstrated that autophosphorylation of the sensor kinase RegB *in vitro* is inhibited by the addition of oxidized coenzyme Q<sub>1</sub>, whereas reduced coenzyme Q<sub>1</sub> exhibits no inhibitory effect on kinase activity. In contrast to those redox signaling processes, in which quinones provide the oxidative power under aerobic conditions, we now revealed strong evidence that in *K. pneumoniae* the reducing power of menaquinones is involved in redox signaling for *nif* gene regulation. We further propose that the electron transfer from the menaquinone pool to NifL occurs by direct contact of reduced menaquinone and the regulatory protein. However, specific menaquinone binding sites, which are known for transmembrane domains of components of respiratory complexes (61–63), have not been identified yet in NifL. At the current experimental status we cannot completely exclude that NifL reduction also occurs by electrons derived from the ubiquinone pool, which might be in equilibrium with the menaquinone pool by a MQ:UQ oxidoreductase in *K. pneumoniae*. However, under aerobic conditions, this will not allow sequestration of NifL to the cytoplasmic membrane because reduced NifL will be rapidly re-oxidized by molecular oxygen. Linking the regulatory mechanism of NifL to the anaerobic respiratory chain and the reducing power of the respective menaquinone pool (see Fig. 1) further allows the possibility of integration of the energy status of the cell in addition to the signal of oxygen status. This is particularly attractive as nitro-

gen fixation is a high energy and high reducing power consuming process (46).

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