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On the role of flavin in malate oxidation by *Azotobacter vinelandii* respiratory membranes

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Flavin oxidation state of the *Azotobacter vinelandii* electron transport fraction containing respiratory membranes was observed with fluorescence spectroscopy. Addition of malate to an anaerobic preparation did not result in flavin reduction. Malate addition to an aerobic preparation slowly reduced flavin, and this reduction was inhibited with addition of 1 mM potassium cyanide or 0.05 mM NAD. Absorption difference spectroscopy was used to observe the oxidation state of both flavin and cytochromes. Addition of malate to an anaerobic preparation reduced the particle cytochromes. Flavin was not reduced unless oxygen was added to the preparation. Pyruvate slowly reduced flavin in the preparation. No evidence could be obtained for pyruvate formation from malate and oxalacetic acid as an explanation for the oxygen-dependent reduction of flavin by malate.

Introduction

The *Azotobacter* have exceptionally high respiration rates relative to other microorganisms [1]. The respiratory system is thought to have a critical role in protecting the oxygen-labile nitrogenase enzymes from destruction by oxygen [2,3]. At high oxygen concentrations, respiration becomes inefficient and the internal oxygen concentration is kept low. For these reasons, the respiratory pathways and their components have been objects of extensive investigation.

Electron transport pathways involving both flavin and cytochromes in *Azotobacter vinelandii* membranes have been proposed [2,4]. Much of this research was done with crude preparations of membrane particles, prepared by differential centrifugation. Many of the conclusions were drawn from results of absorption spectroscopy, observing changes at cytochrome and flavin absorption wavelengths. Addition of malate results in absorption loss at flavin wavelengths [5]. This was attributed to reduction by malate. It has therefore been proposed that the malate-oxidizing activity of the membranes has a flavin component. Here the putative role of flavin was addressed using both fluorescence and absorption spectroscopy to observe reductive changes. Fluorescence spectroscopy has the advantages of observing the reduction/oxidation of flavin in the absence of interference by cytochrome absorption, and because of the sensitivity of fluorescence, at much lower flavin (particle) concentrations. Flavins generally fluoresce in the fully oxidized state but not the one- or two-electron reduced states. This allows the fully oxidized form to be distinguished from the two reduced forms.

Materials and methods

Bacterial growth

Azotobacter vinelandii (ATCC 13705) was cul-

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tured on B6 medium [6] with nitriloacetic acid omitted. Initial pH was 7.5 (KOH). It was grown in 1.5 l of medium in 2.8 l Fernbach flasks on a gyratory shaker at 30 °C. Bacteria were harvested when the A_{580} was 0.35–0.55 (maximum A_{580} was about 0.9).

Membrane particles

Preparation of the R3 electron transport fraction [5] containing respiratory membrane particles was similar to methods used extensively by others (see Refs. 2 and 5). The bacterial wash buffer was 10 mM potassium phosphate (pH 7.5). The disruption and assay buffer was 50 mM potassium phosphate (pH 7.5). Procedures were performed aerobically at 0–4 °C. Bacteria were harvested by centrifugation and washed three times by resuspending and centrifuging (10 min at $12000 \times g$). Washed bacteria were suspended in a small volume of disruption buffer and the suspension sonicated 5×20 s at 90% power with the small probe of a Bronwill Biosonik sonicator. The suspension was centrifuged 30 min at $27000 \times g$ and the pellet discarded. The resulting supernatant was centrifuged 2 h at $144000 \times g$. The pellet from this high-speed centrifugation was rinsed twice with assay buffer and suspended with a small volume of the same buffer. This particle suspension was made anaerobic with argon, stored on ice, and used within 4 h in fluorescence studies. For absorption studies, washed bacteria were depleted of endogenous reductant by stirring overnight in assay buffer at 4 °C [7]. The depleted bacteria were washed and disrupted as described above and the final particle suspension frozen and stored at −80 °C. Upon thawing, the suspension was clarified by brief sonication. It was then made anaerobic with argon and stored at 4 °C until used. The freezing and thawing had no detectable effect on NAD(P)H- or malate-supported oxygen uptake.

Oxygen uptake

Particle-catalyzed oxygen uptake was measured with a Clarke-type electrode in a water-jacketed chamber (Gilson Medical Electronics, Middleton, WI) at 23 °C.

Spectroscopy

Fluorescence spectroscopy was performed with a Spex Fluoralog series 111C spectrofluorometer with a 150 W xenon lamp. Experiments were done at 22 °C using a 3.0-ml membrane suspension in assay buffer with a special cuvette apparatus designed for anaerobic spectroscopy (Fig. 1). The cuvette has a volume of 4 ml and a path length of 1 cm. Additions of anaerobic reagents were made without introduction of extraneous oxygen (legend, Fig. 1). Flavins were measured with excitation at 452 nm and emission at 520 nm. Excitation and emission band passes were 2.25 nm.

A Cary model 210 dual-beam spectrophotometer was used for recording cytochrome and flavin absorption difference spectra. Semi-micro cells (1-cm path length) were used, and each contained 1.0 ml of particle suspension in assay buffer. The anaerobic methods were as described for fluorescence measurements. Spectra were recorded at 22 °C, 1 nm/min scan rate, and 1-nm band pass.

Chemical measurements

Protein was measured by the method of Lowry

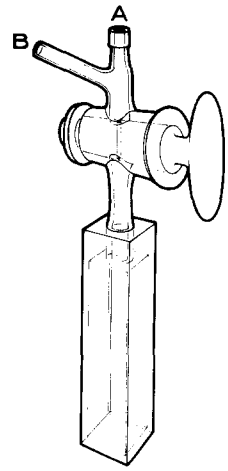

Fig. 1. Anaerobic cuvette apparatus. The cuvette is made anaerobic by repeated evacuation and flush with argon or by inserting a syringe needle into the cuvette and flushing with argon. Solutions are introduced anaerobically with syringes. This procedure involves flushing with argon through port 'B' while opening the stopcock and introducing the syringe needle against the flow of argon. The ground-glass fitting between cell and stopcock is sealed with a small amount of SWAK. Standard stopcock grease is used in the stopcock.

et al. [8] after precipitation with trichloroacetic acid. Oxalacetic acid and pyruvate were determined by reacting with 2,4-dinitrophenylhydrazine as described [9]. Spectra were recorded to distinguish the products.

Reagents

Na_2NADH, NAD, FMN, oxalacetic acid, sodium pyruvate, L-malic acid, sodium DL-lactate and 2,4-dinitrophenylhydrazine were purchased from Sigma Chem. Co., St. Louis, MO. Potassium cyanide and sodium hydrosulfite were purchased from Fisher Scientific Co., Fairlawn, NJ. Where appropriate, stock solutions of reagents were made up in the assay buffer. Malate stock solutions were titrated to pH 7.5 with KOH. Dithionite stock solutions were made up anaerobically, assuming purity to be 100%. SWAK (anaerobic cuvette sealant (Fig. 1)) was a product of Cajon Co., Macedonia, OH. Argon and oxygen were purchased from a local supplier. The argon was purified of the last traces of oxygen with the apparatus described earlier [10]. All other chemicals were reagent grade.

Results

Malate reduction of electron carriers

The respiratory activity supported by malate and other reductants with this electron transport fraction has been described [2]. Oxygen uptake rates, with substrates and inhibitors at concentrations used in these studies are presented in Table I. The rate with 5 mM malate is substantial.

Fig. 2 shows the effects of two different malate treatments on flavin fluorescence of the electron transport fraction. For comparison, the effects of dithionite are also presented. The initial fluorescences are the same and are presented as one tracing. In each case, the cuvette was removed from the spectrofluorometer (fluorescence decrease to zero at about 2 min) and the additions made. The cuvette is replaced in the spectrofluorometer and the results of the treatment on the fluorescence are observed. Malate addition to an anaerobic preparation caused only a very slight decrease of the fluorescence and so did not significantly reduce the flavin. Dithionite did. When the suspension was initially aerobic, however, the fluorescence decreased several minutes after malate addition. This indicated that flavin was reduced. The final fluorescence level observed was substantially more than that obtained with dithionite as reductant, indicating that flavin was only partially reduced. Spectra were recorded before and after the reduction by malate and the calculated difference spectra were essentially the same as those

TABLE I

EFFECT OF SUBSTRATES AND INHIBITORS ON OXYGEN UPTAKE BY THE *A. VINELANDII* ELECTRON TRANSPORT FRACTION

Data represents mean ± S.E. for three determinations. Rates without added substrate were zero.

Additions	Oxygen uptake rate (μmol/min per mg protein)
5.0 mM L-malate	0.519 ± 0.015
+1 mM KCN [a]	0.005 ± 0.001
+0.05 mM NAD [b]	0.551 ± 0.030
+0.05 mM NADP [b]	0.544 ± 0.062
0.05 mM NADH	0.219 ± 0.009
0.05 mM NADPH	0.016 ± 0.001
5.0 mM pyruvate	0.002 ± 0.001

[a] KCN added after malate, when 30% of the O_2 was depleted.
[b] Additions made 4 min prior to malate.

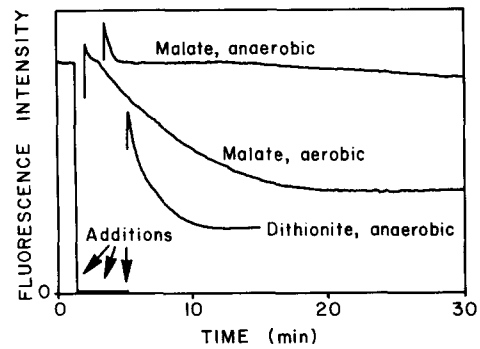

Fig. 2. The effects of malate on flavin fluorescence in the *A. vinelandii* electron transport fraction. Anaerobic particles were reduced with addition of either 5 mM malate or 0.25 mM dithionite. An air-equilibrated suspension was reduced with 5 mM malate. The initial fluorescences were the same and the individual traces show the results when the spectrofluorometer cuvette was replaced in the spectrofluorometer, just after the additions to the cuvette. Each cuvette contained 0.26 mg/ml protein. Full scale fluorescence equals that of 83 μM FMN.

of unreduced FMN. This confirmed the flavin nature of the malate-altered fluorescence. The reductions by malate and dithionite were slow compared with reduction by NAD(P)H. Addition of either 0.05 mM NADH or 0.05 mM NADPH to the anaerobic preparation gave a much more rapid reduction of flavin to a level intermediate between that of dithionite and malate-reduced flavin (data not presented). The reduction was almost completed when the cuvette was replaced in the spectrofluorometer.

Reduction of flavin by addition of malate to the aerobic suspension (Fig. 2) indicated that electron transport to oxygen might be required for the reduction. To investigate this possibility, the terminal oxidase inhibitor cyanide was used. Cyanide partially blocked the reduction of flavin that occurred (Fig. 3). Cyanide strongly inhibited malate-dependent oxygen uptake (Table I).

Reduction of flavin resulting from electron transport might occur as a result of side reactions, conceivably mediated by low levels of contaminating pyridine nucleotides. This possibility was examined by performing the malate-dependent aerobic reduction in the presence of a low concentration of NAD(P) (Fig. 4). NAD strongly inhibited the flavin reduction instead of stimulating it, whereas NADP gave a slight inhibition. Neither compound significantly affected the malate-dependent oxygen uptake (Table I).

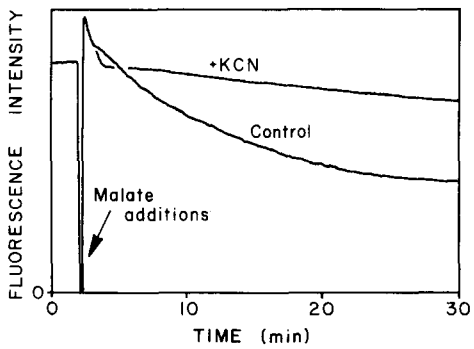

Fig. 3. The effect of cyanide on aerobic reduction of flavins of the *A. vinelandii* electron transport fraction by malate. Potassium cyanide (1 mM) was added 2 min prior to the beginning of the experiment. The initial fluorescences with and without the cyanide were the same and are presented as one tracing. The cuvettes contained 0.23 mg/ml protein. Other conditions as in Fig. 2.

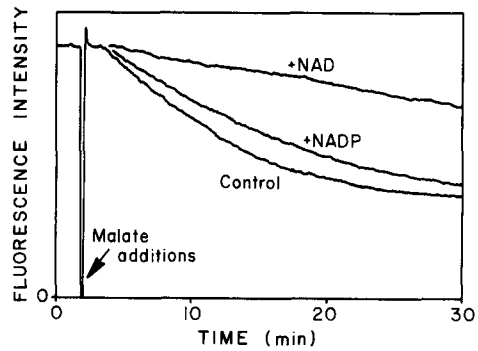

Fig. 4. The effects of NAD(P) on the aerobic reduction of flavins of the *A. vinelandii* electron transport fraction by malate. Either NAD (0.05 mM) or NADP (0.05 mM) were added 5 min prior to the beginning of the experiment. The tracings differed only after malate additions and are presented as only one tracing initially. The cuvettes contained 0.19 mg/ml protein. Full scale fluorescence equals that of 53 μM FMN.

The observations of electron carriers were extended to cytochromes with the use of absorption spectroscopy. In all the experiments, the sample cuvette was initially anaerobic and additions were made as indicated. The reference cuvette was kept air-equilibrated. Traces A, B, and C in Fig. 5 show the difference spectra of anaerobic (no additions) minus aerobic suspensions 2, 24 and 72 min after preparation of the cuvettes. There is clearly a slow reduction of cytochrome *c* (522 and 552 nm [11]), cytochrome *a* (602 nm [11]) and cytochrome *d* (630 nm, trough at 650 nm [12]). The trough at 460 nm is likely due to cytochromes (see discussions below).

Malate was added to a freshly-prepared anaerobic suspension. The cytochromes were strongly reduced 2 min after addition (trace D, Fig. 5). Cytochrome *b* (531 and 560 nm [11]) was not reduced without the malate addition (compare traces C and D). There is a trough at about 459 nm, which would be expected from loss of flavin absorption after reduction in the sample cuvette. The shape of the trough, however, is as expected for the absorbance decrease observed with reduction of some purified cytochromes [4]. Since little anaerobic reduction of flavin was observed with fluorescence, the trough was probably due almost entirely to cytochrome and not flavin reduction. Trace E was recorded 24 min after the anaerobic malate addition and there was little change from

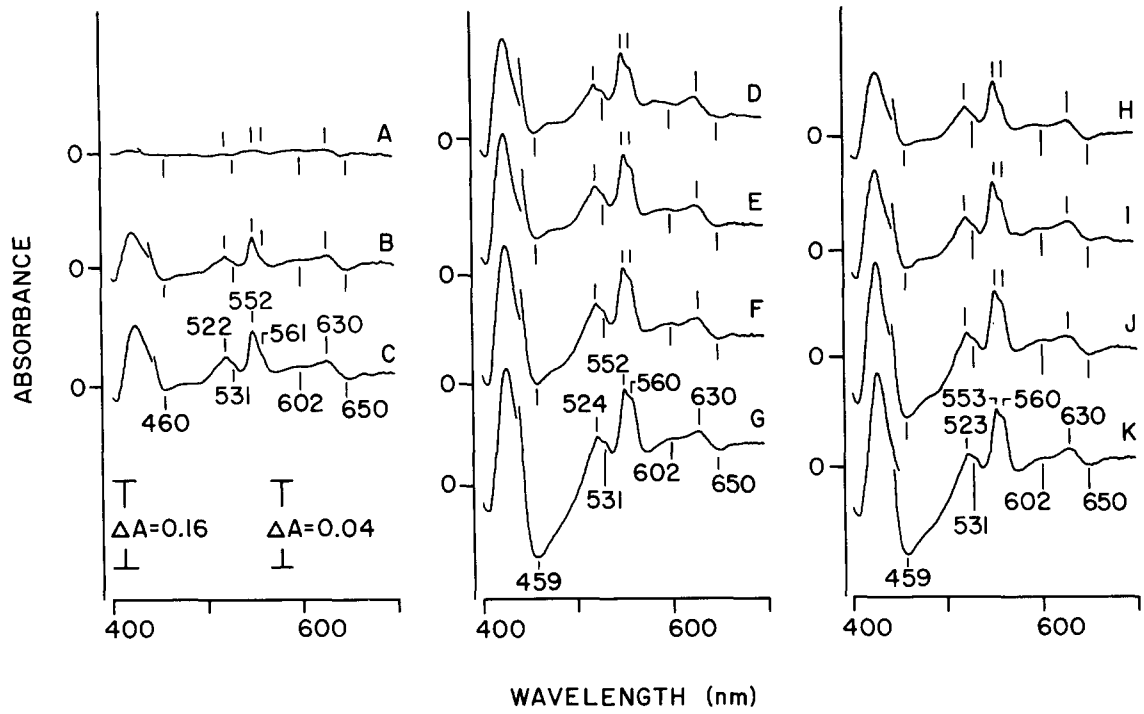

Fig. 5. Reduction of *A. vinelandii* particle electron carriers measured by absorption difference spectroscopy. Traces A–C, D–G, and H–K are from three separate experiments using the same protein preparation. All are anaerobic (argon) minus aerobic suspensions, except as noted. The absorbance scales below trace C apply to all the spectra. Traces A–C have no additions and were recorded after 2, 24, and 72 min. Traces D and E are 2 min and 24 min after malate (5 mM) addition to the sample cuvette. Oxygen was then introduced to the cuvette and trace F is 16 min after the cuvette contents were mixed. The cuvette head space was then equilibrated with argon and 0.1 mM dithionite added. After 12 min the cuvettes were re-scanned (trace G). Traces H–K are scans 12, 24, 48, and 72 min after addition of pyruvate (5 mM) to the anaerobic sample cuvette. Protein concentration in all these assays was 2.34 mg/ml.

the 2 min scan. Oxygen (0.2 ml) was then added to the head space above the suspension. The gas and liquid phases of the cuvette contents were equilibrated by mixing thoroughly and after 16 min the difference spectra recorded. The resulting spectrum (trace F) was essentially the same as before oxygen addition except that the trough at about 459 nm was substantially lower. Since there was no further reduction of cytochromes, it is concluded that the further absorption loss was due to flavin reduction. Subsequent scans at 12 min intervals showed further slight decreases in absorption at 459 nm (not presented). The head space of the sample cuvette was then made anaerobic with argon and the suspension further reduced with addition of dithionite (trace G). Again, the most substantial change was a further decrease in the absorbance at 459 nm.

Pyruvate reduction of flavin

Malate-supported oxygen reduction would be expected to produce oxalacetate from the malate. Oxalacetate can autodecarboxylate to pyruvate. The rate of this reaction varies greatly with the medium but can be rapid [13]. If the initial oxygen concentration is 254 micromolar (air-saturated water at 23° C), then there is potential to produce a concentration of at least 0.5 mM pyruvate from the malate oxidized. The reduction of flavin and cytochromes by pyruvate and the formation of pyruvate in the experimental system was therefore investigated.

Addition of pyruvate to an anaerobic suspension of the electron transport fraction reduced flavin (Fig. 6). The reduction, however, was always slower than the reduction observed with malate. Lactate (2 mM) did not give detectable

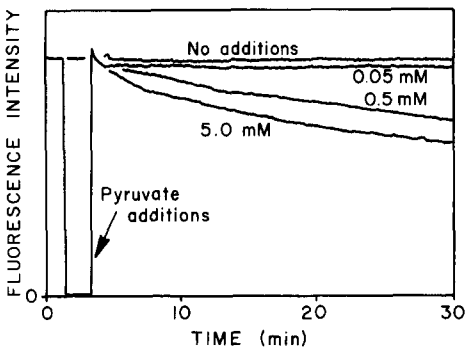

Fig. 6. Pyruvate reduction of flavins of the electron transport fraction of *A. vinelandii*. All assays were anaerobic. Conditions as in Fig. 2. Where no additions were made, the cuvette was not removed from the spectrofluorometer. Final pyruvate concentrations are as indicated and the assays contained 0.28 mg/ml protein.

flavin reduction (data not presented). Reduction by pyruvate was also observed in absorption experiments (Fig. 5). Pyruvate was added to the anaerobic sample cuvette at the initiation of the experiment. Traces H, I, J, and K are spectra recorded 12, 24, 48 and 72 min after pyruvate addition. The cytochromes and flavin were reduced but *b*-type cytochrome and flavin reductions were slower than reductions of cytochromes *c*, *d* and *a*. Pyruvate supported only a very slight oxygen uptake (Table I).

The production of pyruvate from malate and oxalacetate was studied to determine if the quantity necessary for flavin reduction is formed in these experiments. Both pyruvate and oxalacetate can be measured by reacting with 2,4-dinitrophenylhydrazine to form the respective phenylhydrazone [9]. The products absorb at visible wavelengths and can be distinguished by their spectra. Malate does not react. Oxalacetate (0.5 mM) was incubated under the same conditions (pH, buffer, and temperature) as the spectrophotometric experiments. There was no detectable pyruvate formed in 30 min (data not presented). Malate was added to an aerobic suspension of the electron transport fraction in an experiment analogous to the spectrofluorometer experiments. The product formed was oxalacetate (about 0.5 mM) without any detectable pyruvate (data not presented). It is therefore concluded that pyruvate formed from autodecarboxylation of oxalacetate is insufficient to cause the observed reduction of flavin.

Discussion

Previous investigations have yielded data suggesting that the malate-oxidizing activity of *Azotobacter vinelandii* respiratory membranes has a flavin component [5]. The data were from absorption spectroscopy and the effects of added flavins on oxygen uptake. In the absorption experiments described herein, malate reduction of the particle preparation resulted in changes at flavin wavelengths that might be attributed to flavin reduction. Fluorescence loss, which is a more specific indicator of flavin reduction, was very slight under anaerobic conditions. The changes in absorption were therefore not due to direct reduction of flavin by malate but to other mechanisms. One of these is the absorption loss that results from cytochrome reduction. This absorption loss is from shifts of the Soret peak and can easily be mistaken for flavin.

The other mechanism for an apparent malate-dependent flavin reduction appears to require electron transport through the membrane system. When malate is added to an aerobic preparation, reduction of flavin occurs. There is a lag before flavin reduction. Comparison of the oxygen uptake rates (Table I) with the duration of the lag indicates that the malate is reducing all the oxygen during this lag period. The flavin reduction apparently occurs when the particles become anaerobic. The inhibition of reduction by cyanide supports these ideas. The inhibition by NAD suggests an involvement of enzyme(s) of pyridine nucleotide metabolism, although it also indicates that pyridine nucleotides do not mediate the electron transfer. The lack of an effect of NAD on malate-dependent oxygen uptake further indicates that the malate-dependent flavin reduction depends on anaerobic conditions achieved by the malate-dependent oxygen uptake. The exact mechanism of this electron transport-dependent flavin reduction is not known. It could perhaps involve other proteins of high molecular weight that sediment with membranes during high-speed centrifugation [14]. Alternatively, it could be a high potential flavoprotein such as that proposed for

Bradyrhizobium japonicum membranes [15]. It is clear, however, that these spectral data provide no evidence for a direct involvement of flavin in malate oxidation. Furthermore, these experiments demonstrate the usefulness of combining absorption and fluorescence spectroscopy in this type of study.

Malate-dependent oxygen uptake by *A. vinelandii* respiratory particles is enhanced by added FAD [5]. This result suggests an involvement of flavin in malate oxidation. If this flavin cycled between semiquinone and hydroquinone forms during electron transfer, it would not be observable in the spectroscopy experiments described herein.

Acknowledgement

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