REACTIVITY OF MEMBRANEOUS CYTOCHROME OXIDASE (CYTOCHROME aa_3) FROM *ANACYSTIS NIDULANS* TOWARDS *c*-TYPE CYTOCHROMES

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1. Introduction

Oxidation of mammalian cytochrome *c* by cell-free preparations of cyanobacteria has occasionally been observed [1–5]. However, spectral characterization of the terminal oxidase present in some species has been achieved only recently, suggesting the enzyme to be cytochrome aa_3 [6–8]. Other cytochromes, notably the *b*- and *c*-type cytochromes involved in photosynthesis [9,10], have not been shown so far to participate in cyanobacterial respiratory electron transport in vivo. Yet there are indications that in some species plastoquinone [11–13] and, in membrane preparations of *Anabaena variabilis*, plastocyanin and cytochrome *c*-553 [5] might serve in both photosynthesis and respiration. It remains unclear, however, to which extent respiratory and photosynthetic electron transport assemblies actually are identical in cyanobacteria [14–16]: Evidence has recently been obtained that in intact *A. nidulans* part of the respiratory electron flow [6,17] and, in particularly, of the cytochrome oxidase (Peschek, G. A., Muchl, R. and Schmetterer, G., unpublished) might be associated with the (chlorophyll-free [18]) cytoplasmic membrane.

Cytochrome aa_3 is known as a terminal oxidase in several bacteria [19–23]. However, besides the enzyme from *Paracoccus denitrificans* [20], bacterial oxidases usually show modest, if not poor, reactivity towards mammalian cytochrome *c* [21,23,24]. Molecular properties (viz. subunit composition) of bacterial aa_3-type enzymes have been found to be quite different from mammalian cytochrome oxidase [21,23,25]; thus differences in their reaction with cytochrome *c* would not be surprising [24]. Cyanobacterial aa_3-type oxidases have not yet been obtained in a purified state, and direct information on molecular details is lacking.

The present report describes the reaction of membraneous cytochrome aa_3 from *A. nidulans* with various *c*-type cytochromes (see table 1), including horse heart cytochrome *c* and cytochromes c_2 from phototrophic bacteria, using both spectrophotometric and polarographic assays. Rates of oxidation of the various cytochromes *c* were found to parallel those of cytochrome aa_3 reduction by the respective cytochromes. For comparison, the NADPH-dependent cytochrome *c* reductase activity of the *Anacystis* membranes was also measured. Increasing ionic strength of the assay medium progressively depressed the oxidation of all the cytochromes *c* tested. Attempts to study the reactivity of cytochromes *c* from *A. nidulans* [26] towards the cytochrome oxidase of this organism are currently under way in our laboratory.

The results obtained so far suggest a possible homology of the *Anacystis* aa_3-type enzyme to *P. denitrificans* and even mammalian cytochrome oxidases. Moreover, the striking reactivity of the membrane-bound oxidase towards cytochromes c_2 would be consistent [24] with a direct evolution from (anaerobic) phototrophic bacteria to (aerobic) cyanobacteria [27,28].

2. Materials and methods

Anacystis nidulans (strain L-1402-1, Göttingen) was grown axenically at 40°C, and respiratory mem-

Abbreviations: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; PMS, phenazine methosulfate

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branes were isolated as previously described [8]. Presence of cytochrome aa_3 in the membranes was verified spectrophotometrically [6–8], its amount was determined by the use of a $\Delta\epsilon$ (ascorbate-reduced minus ferricyanide-oxidized) of 24.0 mM^{-1} cm^{-1} [29]. The cytochrome oxidase reaction was assayed either polarographically or spectrophotometrically, according to standard procedures [30], using a Clark-type oxygen electrode and a Perkin-Elmer dual wavelength spectrophotometer, model 557, respectively. Membrane particles were suspended in solutions containing 0.6 M mannitol, 1 mM TMPD, 20 mM Na-ascorbate and 25 mM K-phosphate buffer, pH 7.5 (polarographic assay; 2.5 ml) or 0.6 M mannitol and 10 mM K-phosphate buffer, pH 7.0 (spectrophotometric assay; 1.0 ml). Reactions were started by addition of 10 μM of the desired cytochrome *c*; this amount proved to saturate the reactions investigated. Assay temperature was 35°C for the reduction or oxidation of cytochromes *c*, and 25°C for the reduction of cytochrome aa_3.

Reduction of the membrane-bound cytochrome aa_3 by the different *c*-type cytochromes was followed anaerobically at 605 minus 590 nm [31] in Thunberg cuvettes containing N_2-flushed membrane suspensions together with 20 mM Na-ascorbate; after isolation of the membranes the native oxidase remained in a fully oxidized state (no further decrease in optical density at 605 nm upon addition of ferricyanide) even under N_2 and in presence of ascorbate until the cytochromes were added [8,16]. Oxidation of reduced cytochromes *c* was followed at the wavelength of the reduced α-peak minus wavelength of the isosbestic point (around 540 nm) which was determined separately for each cytochrome *c* using ascorbate for reduction and ferricyanide for oxidation. For convenience, a differential absorption coefficient of 20 mM^{-1} cm^{-1} was assumed for each cytochrome *c*. The pseudo-first order rate constant of the oxidase reaction (with respect to cytochromes *c*) was calculated according to [32]. Preparative reduction and oxidation of the cytochromes *c* was performed with excess Na-ascorbate and ferricyanide, respectively, followed by dialysis against 31 of 10 mM K-phosphate buffer, pH 7.0, for 36 h at 4°C including three changes of the buffer.

Horse heart cytochrome *c* (Type VI) was obtained from Sigma, *Candida krusei* cytochrome *c* was from Calbiochem. The other cytochromes *c* were a generous gift of Drs G. Hauska and W. Lockau, Regensburg, FRG. All chemicals used were of the highest purity available.

3. Results

Fig.1 shows a reduced minus oxidized difference spectrum (A) and the corresponding carbon monoxide difference spectrum (B) of the *Anacystis* membrane preparation recorded in the region of the α-band of *a*-type cytochromes. The rather broad peaks at 605 and 590 nm, respectively, suggest the presence of cytochrome aa_3 [6–8]. The concentration of cytochrome aa_3 was 0.07–0.1 nmol/mg membrane protein.

Table 1 gives the rate of the cytochrome oxidase reaction with each of the eight different *c*-type cytochromes tested (polarographic measurements). Wavelengths of the reduced α-peak and iso-electric points of the cytochrome *c* proteins are included in the table. Effects of the detergent Tween 80 and the inhibitor KCN are also shown. The reactivity pattern revealed by O_2 uptake measurements (table 1) entirely paralleled the one observed in the spectrophotometric assay (fig.2); minor quantitative deviations obviously resulted from different assay conditions [30,33]. In both types of assay the rate of reaction between the *Anacystis* cytochrome oxidase and the cytochromes *c* decreased in the following order (source organism of the *c*-type cytochrome): *Rhodopseudomonas*

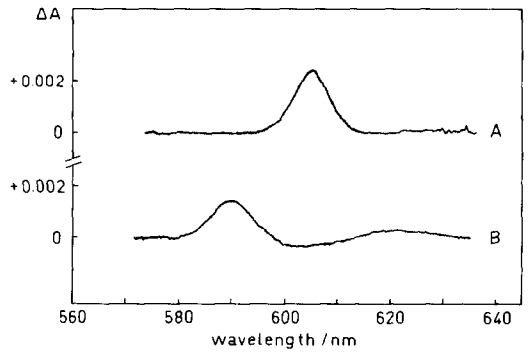

Fig.1. (A) Ascorbate plus PMS reduced minus ferricyanide oxidized spectrum of the *Anacystis* membranes recorded between 580 and 640 nm (α-region); (B) ascorbate plus PMS reduced plus carbon monoxide minus ascorbate plus PMS reduced spectrum. Membrane particles (1 mg protein/ml) were suspended in 25 mM K-phosphate buffer, pH 7.5, containing 0.8 M mannitol and measured at 4°C in a fully computerized Perkin-Elmer spectrophotometer, model 557.

Table 1
Polarographic cytochrome oxidase assay using different *c*-type cytochromes

Cytochrome *c* (source organism)	Reduced α-peak (nm)[a]	I.E.P. [Ref.]	Oxygen uptake (nmol per min per mg protein)[b]		
			–	+0.05% Tween 80	+5 μM KCN
1 *Rhodopseudomonas spheroides*	550	4.9 [38]	420	830	20
2 *Rhodopseudomonas capsulata*	550	4.7 [38]	310	600	15
3 *Rhodopseudomonas viridis*	551	–	85	160	4
4 Horse	550	10.0 [35]	45	95	2
5 *Candida krusei*	549	>7 [35]	25	50	1
6 *Anabaena variabilis*	553	>7 [5]	15	35	1
7 *Euglena gracilis*[c]	552	5.5 [35]	0	0	0
8 *Scenedesmus obliquus*[c]	553	<7 [35]	0	0	0

[a] Position of α-peak of ascorbate-reduced cytochromes
[b] Rates corrected for O_2 uptake in the absence of membranes (<5 nmol/min throughout); Tween 80 and KCN were added to the membrane suspension 3 min before starting the measurements by addition of the desired cytochrome *c* (10 μM final concentration)
[c] Plastidic ('photosynthetic') cytochromes

Oxygen uptake by 2.5 ml of the *Anacystis* membrane suspension containing 7–13 mg protein (80 pmol cytochrome aa_3/mg) was followed with a Clark-type oxygen electrode at 35°C in the dark. For details see section 2. I.E.P. = iso-electric point

sphaeroides > *R. capsulata* > *R. viridis* > *horse* > *Candida krusei* > *Anabaena variabilis* > *Euglena gracilis* (plastidic) > *Scenedesmus obliquus* (plastidic).

In order to confirm that the cytochromes *c* were all oxidized through the aa_3-type oxidase present in the *Anacystis* membranes the rate of reduction of the enzyme was measured directly at 605 minus 590 nm [31]. Starting with initially fully oxidized enzyme the

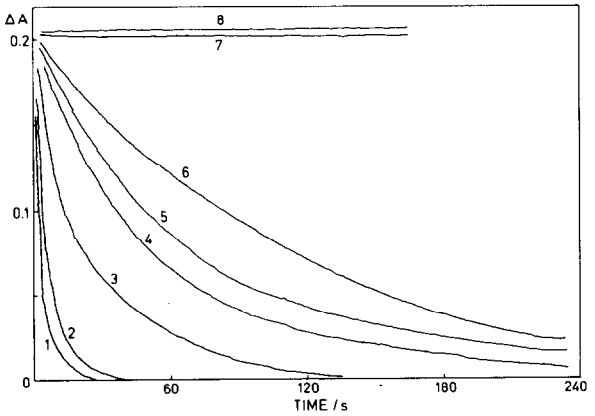

Fig.2. Spectrophotometric cytochrome oxidase assay using different *c*-type cytochromes. Numbers correspond to the cytochromes as listed in table 1. Cuvettes contained 0.144 mg membrane protein/ml, equivalent to about 14 pmol cytochrome aa_3 per cuvette. Oxidation of the reduced cytochromes *c* was followed by the decrease in ΔA measured between the wavelength of the reduced α-peak minus the wavelength of the isosbestic point of the respective cytochromes. For details see section 2.

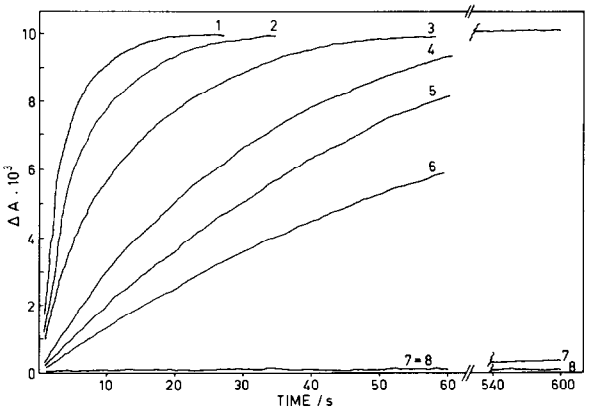

Fig.3. Reduction of the membrane-bound aa_3-type oxidase of *A. nidulans* in the presence of different reduced *c*-type cytochromes. Reduction of the oxidase was followed by the increase in ΔA measured between 605 and 590 nm [31]. Assays contained 5.3 mg protein/ml (70 pmol cytochrome aa_3/mg protein). Reactions were started by addition of the respective cytochromes *c* (10 μM) in the presence of excess ascorbate (see section 2). Numbers correspond to the cytochromes as listed in table 1.

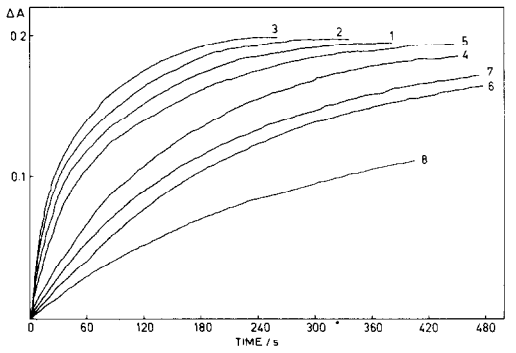

Fig.4. Spectrophotometric cytochrome *c* reductase assay using different *c*-type cytochromes. Numbers correspond to the cytochromes as listed in table 1. Reduction of the oxidized cytochromes *c* in presence of 5 mM NADPH as the physiological electron donor [11,16] was followed by the increase in ΔA measured between wavelength of the reduced α-peak minus wavelength of isosbestic point of the respective cytochromes (for details see section 2). No reaction between NADPH and the cytochromes *c* occurred in the absence of membranes, nor did the membranes reduce the cytochromes in the absence of NADPH.

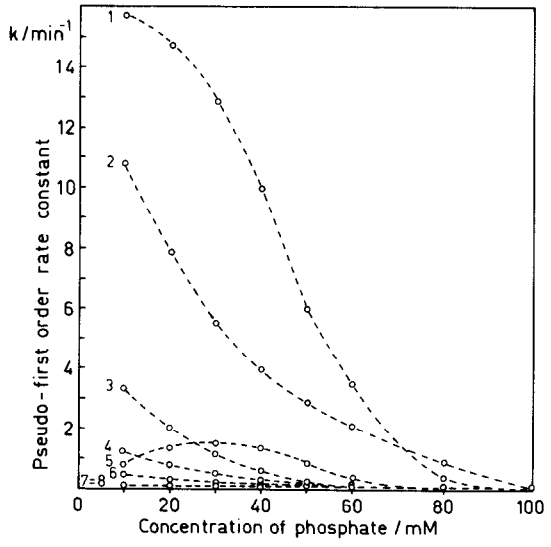

Fig.5. Effect of increasing phosphate buffer concentrations on the spectrophotometrically assayed cytochrome oxidase activity supported by different *c*-type cytochromes. Numbers correspond to the cytochromes as listed in table 1. pH of the buffer was 7.0 at each concentration tested. Pseudo-first order constants were calculated according to [32]. For details see section 2. Increasing concentrations of NaCl in 10 mM phosphate buffer gave qualitatively similar results (not shown).

reduced *c*-type cytochromes were allowed to react anaerobically so as to prevent reoxidation of the aa_3-enzyme by O_2 (see section 2). It is seen from fig.3 that the rate of reduction of the oxidase by the different cytochromes *c* reflected the order of their differential reactivities in the conventional cytochrome oxidase assays (table 1 and fig.2).

When, instead of cytochrome *c* oxidase, the NADPH-dependent cytochrome *c* reductase of the membranes [11,16] was assayed, the reactivity pattern observed was quite different, then following the order (source organisms of cytochromes *c*) *R. viridis* > *R. capsulata* > *R. sphaeroides* > *C. krusei* > horse > *E. gracilis* > *A. variabilis* > *S. obliquus* (fig.4).

Fig.5 shows the effect of increasing concentrations of the phosphate buffer on the oxidation of different *c*-type cytochromes. As exemplified by other systems [5,21] high ionic strength appeared to weaken the interaction between cytochromes *c* and the membrane-bound *Anacystis* oxidase, thereby leading to decreased reaction rates.

4. Discussion

Respiratory electron transport is a rather neglected field of research among an otherwise highly developed 'cyanobacteriology' [15,16]. However, the recent discovery of cytochrome aa_3 as a functional respiratory cytochrome oxidase in cyanobacteria [6–8] and new results concerning possible composition [5,11–13,16] and localization [6,17] of the cyanobacterial respiratory chain may permit new approaches to an old problem.

The present paper gives an account of the reaction between membraneous aa_3-type oxidase from *A. nidulans* and various cytochromes *c*. Similar surveys have been published for other cytochrome oxidases, mostly of aa_3-type, both of mammalian and bacterial origin [21,24,34,35], and also for the d_1c type cytochrome oxidase from *Pseudomonas aeruginosa* [24,35]. The results presented in section 3 now show that, similar to the *Paracoccus denitrificans* enyme [20,23] the aa_3-type cytochrome oxidase of *A. nidulans* might be a major exception to the general rules of reactivity emerging from the studies quoted.

Firstly, apart from an expected inability to oxidize plastidic cytochromes *c* [5,35,36] the reaction with horse heart cytochrome *c* was fairly fast, and so was its reaction with another mitochondrial cyto-

chrome *c* (from the yeast *C. krusei*). This would tend to relate the molecular properties of the *Anacystis* oxidase to those of the *P. denitrificans* enzyme [23]. Yet to what extent the similarity might hold remains to be seen.

The second peculiarity of the *Anacystis* oxidase was its rapid reaction with several c_2-type cytochromes known to participate in both photosynthesis and respiration of Rhodospirillaceae [37]. Neither mammalian nor bacterial cytochrome oxidases were found to react at appreciable rates with cytochromes c_2 [35,38] which, by contrast, were significantly active in the mitochondrial cytochrome *c* reductase assay [34,38] showing that the sites of attack within the respiratory chain are different with respect to oxidation and reduction of the cytochromes *c*, respectively. Our results with *Anacystis* membranes evidently permit to draw similar conclusions (see fig.4).

Yamanaka has advanced the interesting, though not undisputed [39], hypothesis of co-evolution of cytochromes *c* and cytochrome oxidases towards optimal fitting [24]. Thus, finally, from an evolutionary point of view [27,28] it is attempting to speculate that, regarding the surprisingly fast reaction of the *Anacystis* aa_3-type terminal oxidase with cytochrome c_2 from *R. sphaeroides* this phototrophic bacterium might be the more or less direct ancestor of (some?) cyanobacteria. *R. sphaeroides* is the only member of the phototrophic bacteria known to be capable of synthesizing cytochrome aa_3 [40].

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