REFERENCES
Alberty, R. A., & Massey, V. (1954) *Biochim. Biophys. Acta 13*, 347–353.
Chance, B. (1952) *J. Biol. Chem. 194*, 471–481.
Cotton, M. L., & Dunford, H. B. (1973) *Can. J. Chem. 51*, 582–587.
Elion, G. B., Ide, W. S., & Hitchings, G. H. (1946) *J. Am. Chem. Soc. 68*, 2137–2141.
Gibson, Q. H., & Milnes, L. (1964) *Biochem. J. 91*, 161–171.
Hager, L. P., Morris, D. R., Brown, F. S., & Eberwein, H. (1966) *J. Biol. Chem. 241*, 1769–1777.
Harrison, J. E., & Schultz, J. (1976) *J. Biol. Chem. 251*, 1371–1374.
Hewson, W. D., & Hager, L. P. (1979) *J. Biol. Chem. 254*, 3175–3181.
Kimura, S., & Yamazaki, I. (1978) *Arch. Biochem. Biophys. 189*, 14–19.
Libby, R. D., Thomas, J. A., Kaiser, L. W., & Hager, L. P. (1982) *J. Biol. Chem. 257*, 5030–5037.
Liebhafsky, H. A. (1935) *Chem. Rev. 7*, 89–113.
Liebhafsky, H. A. (1939) *J. Am. Chem. Soc. 61*, 3513–3520.
Liu, T. N. E., M'Timkuku, T., Geigert, J., Wolf, B., Neidleman, S. L., Silva, D., & Hunter Cevera, J. C. (1987) *Biochem. Biophys. Res. Commun. 142*, 329–333.
Manthey, J. A., & Hager, L. P. (1981) *J. Biol. Chem. 256*, 11232–11238.
Manthey, J. A., & Hager, L. P. (1985) *J. Biol. Chem. 260*, 9654–9659.
Manthey, J. A., McElvaney, K. D., & Hager, L. P. (1984) *Methods Enzymol. 107*, 439–445.
Morris, D. R., & Hager, L. P. (1966a) *J. Biol. Chem. 241*, 3582–3589.
Morris, D. R., & Hager, L. P. (1966b) *J. Biol. Chem. 241*, 1763–1768.
Neidleman, S. L., & Geigert, J. (1983) *Trends Biotechnol. 1*, 21–25.
Siuda, J. F., & DeBernardis, J. F. (1973) *Lloydia 36*, 107–143.
Stelmaszynska, T., & Zgliczynski, J. M. (1974) *Eur. J. Biochem. 45*, 305–312.
Thomas, J. A., Morris, D. R., & Hager, L. P. (1970a) *J. Biol. Chem. 245*, 3129–3134.
Thomas, J. A., Morris, D. R., & Hager, L. P. (1970b) *J. Biol. Chem. 245*, 3135–3142.
Weisner, W., Van Pee, K. H., & Lingens, F. (1986) *FEBS Lett. 209*, 321–324.
Wever, R., Plat, H., & Hamers, M. N. (1981) *FEBS Lett. 123*, 327–331.

Characterization of the Cytochrome *c* Oxidase in Isolated and Purified Plasma Membranes from the Cyanobacterium *Anacystis nidulans*†

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ABSTRACT: Functionally intact plasma membranes were isolated from the cyanobacterium (blue-green alga) *Anacystis nidulans* through French pressure cell extrusion of lysozyme/EDTA-treated cells, separated from thylakoid membranes by discontinuous sucrose density gradient centrifugation, and purified by repeated recentrifugation. Origin and identity of the chlorophyll-free plasma membrane fraction were confirmed by labeling of intact cells with impermeant protein markers, [^{35}S]diazobenzenesulfonate and fluorescamine, prior to membrane isolation. Rates of oxidation of reduced horse heart cytochrome *c* by purified plasma and thylakoid membranes were 90 and 2 nmol min^{-1} (mg of protein)$^{-1}$, respectively. The cytochrome oxidase in isolated plasma membranes was identified as a copper-containing aa_3-type enzyme from the properties of its redox-active and EDTA-resistant Cu^{2+} ESR signal, the characteristic inhibition profile, reduced minus oxidized difference spectra, carbon monoxide difference spectra, photoaction and photodissociation spectra of the CO-inhibited enzyme, and immunological cross-reaction of two subunits of the enzyme with antibodies against subunits I and II, and the holoenzyme, of *Paracoccus denitrificans* aa_3-type cytochrome oxidase. The data presented are the first comprehensive evidence for the occurrence of aa_3-type cytochrome oxidase in the plasma membrane of a cyanobacterium similar to the corresponding mitochondrial enzyme (EC 1.9.3.1).

Cyanobacteria (blue-green algae) or their immediate ancestors were the first organisms that carried out oxygen-releasing, plant-type photosynthesis in a hitherto essentially anaerobic biosphere (Stanier & Cohen-Bazire, 1977; Broda, 1975). At the same time the oxygen-producing cyanobacteria must have been among the first to cope with free oxygen, a well-known cell poison to strict anaerobes (Morris, 1975), eventually by some type of aerobic respiration making use of preformed photosynthetic electron-transport pathways (Broda & Peschek, 1979). In fact, common electron carriers occur in photosynthesis and respiration of both anoxygenic phototrophic bacteria (Baccarini-Melandri & Zannoni, 1978) and oxygenic cyanobacteria (Aoki & Katoh, 1982; Peschek & Schmetterer, 1982). It is not surprising, therefore, that the cyanobacteria have become the largest and ecologically most successful group of prokaryotes (Stanier & Cohen-Bazire,

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1977). What is more surprising is that cyanobacterial respiration has received only scant attention so far [for review, cf. Peschek (1987)].

Similar to the chloroplasts of higher plants (Douce et al., 1984; Kirk & Tilney-Bassett, 1978), the cyanobacterial cell contains, in addition to the cytoplasmic or plasma membrane (CM),[1] an elaborate network of intracytoplasmic or thylakoid membranes (ICM). Since photosynthesis and respiration share common electron-transport components, the view has been widely held that the thylakoid membranes are the exclusive site of both photosynthetic and respiratory electron flow. However, there are scattered results to be found in the literature that also the plasma membrane of certain cyanobacteria may be a competent site of electron transport (Raboy & Padan, 1978; Reed et al., 1981; Fry et al., 1986; Peschek, 1984; Erber et al., 1986) and that the cytochrome oxidase involved may be of aa_3 type (Matthijs, 1984; Houchins & Hind, 1984; Fry et al., 1985; Häfele et al., 1988). Evidence for this was derived from measurements on intact cells and spheroplasts or crude membranes since until the pioneering work of Murata et al. (1983) there was no reliable way to obtain isolated and separated, yet physiologically fully active preparations of CM and ICM from cyanobacteria. Following our previous work on the isolation and separation of active CM and ICM from *Anacystis nidulans* exhibiting high rates of horse heart ferrocytochrome *c* oxidation (Molitor & Peschek, 1986; Molitor et al., 1986, 1987; Peschek et al., 1988), we thought it worth while to attempt a comprehensive characterization of our CM preparations and of the cytochrome oxidase contained therein. Thus for the first time our data permit the safe conclusion that a cytochrome oxidase of aa_3 type does occur in a cyanobacterium and that this enzyme is contained in both plasma and thylakoid membranes, relative shares critcally depending on growth conditions (Peschek et al., 1988).

Materials and Methods

Growth and Harvest of the Organisms. Anacystis nidulans (*Synechococcus* sp.), strain 1402-1, Göttingen, FRG, was grown photoautotrophically in axenic batch culture [modified medium D of Kratz and Myers gassed wtih 1.5% (v/v) of CO_2 in sterile air; 20–25 $W \cdot m^{-2}$ warm white fluorescent light as measured with a YSI radiometer, Model 65, at the surface of the vessels; 35–38 °C; pH 7.9–8.6] as described previously (Molitor & Peschek, 1986). Purity of the cultures was routinely checked under the phase-contrast microscope or by inoculation of Difco Bacto nutrient agar with a few drops of the culture, followed by a 4-week incubation period at 35 °C. The data presented here were obtained with cultures free of bacterial or other contaminants. Cells were harvested after 2–3 days of growth (final cell density around 3 μL of packed cells/mL of culture) by centrifugation at room temperature (4000*g*, 10 min), washed twice with triple-distilled water ($R > 5$ MΩ), and resuspended in 5 mM NaCl, 2 mM Na_2EDTA, and 10 mM Hepes/NaOH buffer (pH 7.4) to a cell concentration of 80 μL/mL of packed cells (final volume 90–100 mL). *Escherichia coli* (strain RR1, a $recA^+$ derivative of HB101) was a gift of Dr. A. Hartig (Institute of Biochemistry, University of Vienna) and grown as described (Bolivar & Backman, 1979).

Preparation and Purification of Plasma Membranes. The cell suspension was made up to a concentration of 20% (w/w) sucrose and 0.5% lysozyme, incubated at 37 °C under room light for 2 h, and centrifuged at 3000*g* at room temperature for 10 min, yielding a colorless supernatant. Control experiments established that the harvested cells catalyzed normal rates of respiration and photosynthesis (not shown). The pellet was resuspended in the original volume of Hepes/EDTA/NaCl buffer containing 1 mM PMSF and 0.0075% DNase I (from Sigma) and incubated at 20 °C for 10 min. The suspension was passed once through a precooled French pressure cell at 33 MPa (4790 psi) and centrifuged at 5000*g* and 4 °C for 10 min to remove intact cells and cell debris. The blue-green supernatant of the centrifuged cell-free extract containing the membrane vesicles was made up to a concentration of 42% (w/w) with solid sucrose and supplemented with another 1 mM PMSF; 18 mL of this suspension was placed on the bottom of a centrifuge tube, sequentially overlaid with 6, 6, and 5 mL of 35, 30, and 10% (w/w) sucrose, respectively, each fraction containing 10 mM Hepes/NaOH, 2 mM Na_2EDTA, 5 mM NaCl, and 1 mM PMSF (final pH 7.4). Five to six tubes were prepared in this way. Fractionation of the membranes was by flotation centrifugation in a Beckman ultracentrifuge, Model L5-50, using an SW-27 swinging-bucket rotor ($131500g_{max}$; 16 h; 4 °C). Membranes equilibrating at 30 and 35/42% (w/w) sucrose, respectively (cf. Figure 1), were individually withdrawn from the gradient, properly diluted with 10 mM Hepes/NaOH buffer (pH 7.4) containing 5 mM NaCl, 2 mM Na_2EDTA, and 1 mM PMSF, and sedimented by centrifugation at 175000*g* and 4 °C for 45 min. The pellet was resuspended in a small volume of 10 mM Hepes/NaOH buffer (pH 7.4), layered on top of a discontinuous sucrose gradient [4/6/6/18 mL of 10/30/35/42% (w/w) sucrose, respectively, from top to bottom; cf. Figure 1], and run for another centrifugation as before. In this way, each of the two major membrane fractions was recentrifuged severalfold on fresh sucrose density gradients until the yellow membrane fraction was absolutely free of methanol-extractable and spectroscopically detectable chlorophyll, and no further yellow membranes were recovered from recentrifuged green membrane fractions. All centrifugation was done at 4 °C and in the presence of the protease inhibitor PMSF which was added immediately before each step of the procedure. All solutions used were prepared with triple-distilled water.

Identification of the Plasma Membrane. Intact cells were reacted with [^{35}S]DABS (Berg, 1969; Tinberg et al., 1974) or fluorescamine (Weigele et al., 1972; Udenfriend et al., 1972) according to established procedures. After cell breakage and membrane isolation, the radioactivity of fluorescence associated with CM and ICM was measured on a Packard Tricarb liquid scintillation counter, Model 3320, or a Shimadzu spectrofluorometer, Model RF 540. All data were corrected for quenching by membrane pigments with unlabeled membranes as internal quenching standard. Under the conditions tested, fluorescence intensity from protein-bound fluorescamine (λ_{ex} = 390 nm, λ_{em} = 480 nm) and radioactivity from [^{35}S]DABS (counts per minute, cpm) were linear with membrane protein concentration. High specific activities (per milligram of membrane protein) of the membrane-impermeant protein markers were taken to result from plasma membranes (cf. Figure 2).

Identification of the Cytochrome Oxidase. Isolated and purified membranes were subjected to SDS–PAGE and immunoblotting as described previously (Trnka & Peschek, 1986; Molitor et al., 1987; Peschek et al., 1988). Positively specific

[1] Abbreviations: PMSF, phenylmethanesulfonyl fluoride; PMS, phenazine methosulfate; TMPD, *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Hepes, *N*-(2-hydroxyethyl)piperazine-*N′*-2-ethanesulfonic acid; DABS, diazobenzenesulfonate; CM, plasma membrane; ICM, thylakoid membrane; ESR, electron spin resonance.

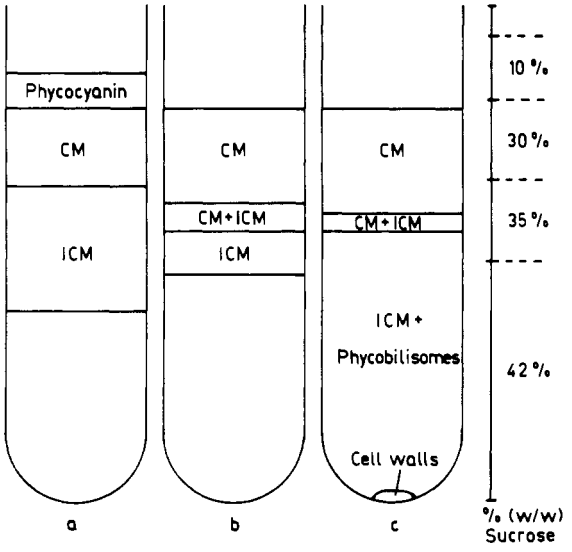

FIGURE 1: Schematic representation of the discontinuous sucrose density gradients containing isolated and separated plasma (CM, yellow) and thylakoid (ICM; green) membranes of *A. nidulans* after flotation centrifugation (c) and purification by conventional recentrifugation (sedimentation) of CM (b) and ICM (a).

and complementary immunological cross-reactions of Western-blotted polypeptides with antibodies against subunits I and II, and against the holoenzyme, of *Paracoccus denitrificans* aa_3-type cytochrome oxidase (Ludwig & Schatz, 1980) indicated the presence of homologous aa_3-type cytochrome oxidase in the *A. nidulans* membranes. No such complementary pattern of cross-reactions (cf. Figure 3) was seen when membrane preparations from *E. coli* were used instead of *Anacystis* membranes (not shown).

Analytical Determinations. Oxidation of reduced horse heart cytochrome *c* (Molitor & Peschek, 1986), oxygen uptake supported by ascorbate-reduced cytochrome *c* (Peschek, 1981), ESR spectra (Fry et al., 1985), reduced minus oxidized optical difference spectra (Peschek, 1981; Peschek et al., 1982), photoaction and photodissociation spectra (Peschek, 1981; Peschek et al., 1982; Appleby, 1969), and spectra of extracted alkaline pyridine ferrohemochromes (Peschek, 1981; Morrison & Hopie, 1965) were determined as described previously. The optical spectra were also determined on 1% octyl glucoside solubilized plasma membranes with essentially identical results but markedly increased sensitivity (Wastyn et al., 1987, 1988). Protein and chlorophyll were determined according to Bradford (1976) and Mackinney (1941), respectively.

Results and Discussion

Plasma Membrane and Cytochrome Oxidase. Figure 1 shows a scheme of the discontinuous sucrose density gradients after centrifugation of the membrane preparations of *A. nidulans*. Initial flotation centrifugation (c) preceded further refinement of individual membrane fractions, still contaminated with each other, by conventional density gradient centrifugation which yielded purified CM (b) and ICM (a). The latter procedure could be repeated several times (purification steps II and III in Figure 2). The small pellet obtained after initial flotation centrifugation probably represents residual cell wall fragments including outer membane; this fraction showed neither cytochrome *c* oxidase activity nor positive cross-reaction with the antibodies (not shown). Figure 2 illustrates how specific labeling of CM and ICM by [^{35}S]DABS (A) or fluorescamine (B) as well as protein-to-chlorophyll ratios (C) and specific cytochrome oxidase activities (D) in the two membrane fractions changed during progressive purification.

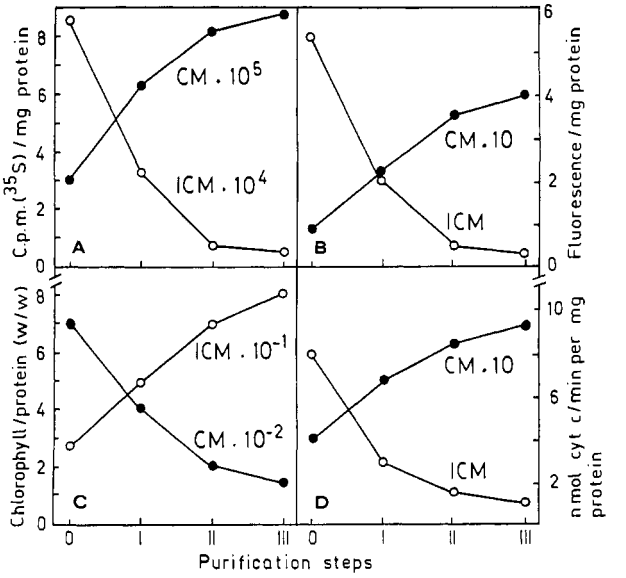

FIGURE 2: Specific radioactivity (A) and fluorescence (B) of CM and ICM preparations from *A. nidulans* labeled with [^{35}S]DABS (A) and fluorescamine (B) and chlorophyll-to-protein ratios (C) and cytochrome oxidase activities (D) of CM and ICM from unlabeled cells at different stages of purification, 0–III (0 corresponds to membranes from flotation gradients; cf. Materials and Methods). Note the different scale used for CM and ICM curves.

This pattern would necessitate the following conclusions: (a) It is the plasma membrane that is preferentially labeled by the external markers; (b) the plasma membrane does not contain a significant amount of chlorophyll; (c) the plasma membrane does contain the major share of cytochrome oxidase when compared to ICM. In addition, the CM of *A. nidulans*, similar to chloroplast envelope membranes (Pineau et al., 1986), may contain chlorophyll precursors such as chlorophyllide *a* and protochlorophyllide *a* (Hinterstoisser et al., 1988). Inhibition profiles of the CM-bound cytochrome oxidase are strikingly reminiscent of the mitochondrial enzyme (Table I). Especially note the very low concentration of KCN sufficient for complete inhibition.

Immunological Cross-Reactivity. Isolated and purified CM and ICM from *A. nidulans* subjected to SDS–PAGE exhibited polypeptide patterns markedly different from each other (Figure 3a). Two of the polypeptides, after Western blotting, gave a positive cross-reaction with (polyclonal) antibodies raised against the aa_3-type cytochrome oxidase from *P. denitrificans* (Ludwig & Schatz, 1980). The cross-reactions were specific and strictly complementary with respect to the antibodies against the holoenzyme (lane 1) and against subunits I (lane 2) and II (lane 3). Apparent molecular weights of the *A. nidulans* cytochrome oxidase subunits I and II were found at 55 000 and 32 000 which are higher by about 10 000 and 4000, respectively, compared to those of the homologous *P. denitrificans* subunits (Ludwig & Schatz, 1980). The latter, in turn, are heavier than the corresponding mammalian cytochrome oxidase subunits I and II by 2500 and 4500 (Höchli & Hackenbrock, 1978). Figure 3b shows that the specific intensity of the cross-reaction (per unit membrane protein) in the ICM decreased with increasing purity (lanes 2a–2c). This indicates that the CM of A. *nidulans* contains not only a more active aa_3-type cytochrome oxidase (cf. Figure 2) but also a higher concentration of this enzyme than ICM.

ESR Signals of Redox-Active Copper. Cytoplasmic membrane preparations exhibited features in the low-temperature ESR spectra at $g = 2.02–2.03$ characteristic of Cu^{2+} in the aa_3-type cytochrome oxidase (Figure 4) (Beinert et al., 1962;

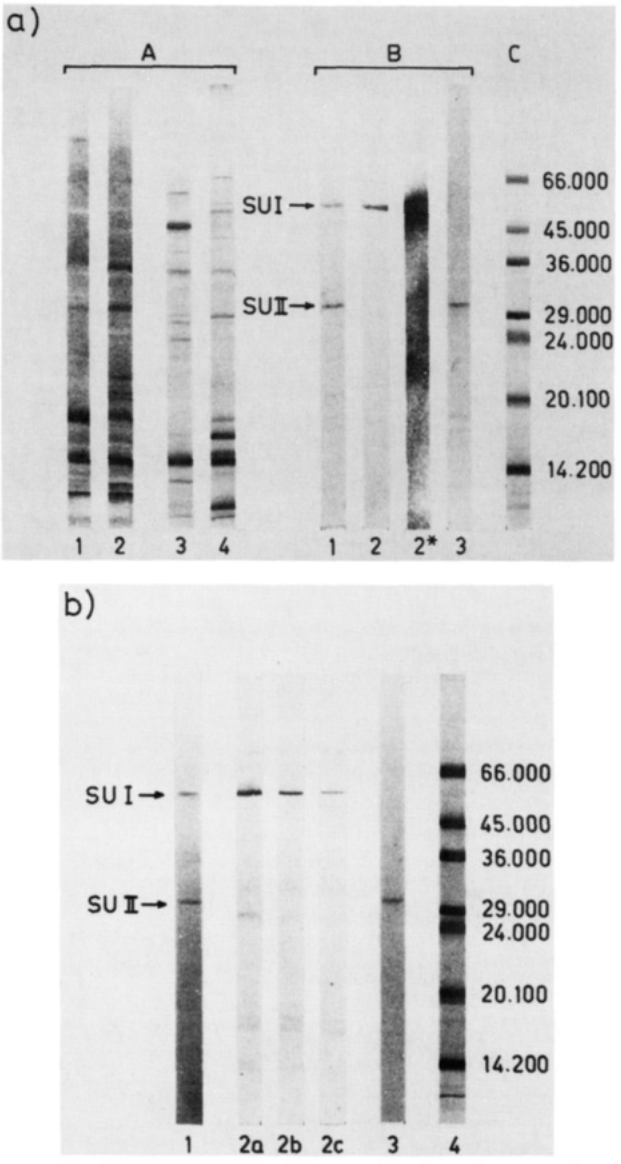

FIGURE 3: (a) SDS–PAGE and immunoblotting of separated and purified membranes from *A. nidulans*. (A) Silver-stained (lanes 1 and 2) and Coomassie blue stained (lanes 3 and 4) CM (lanes 1 and 3) and ICM (lanes 2 and 4) polypeptides, 30 μg of protein was applied to each lane. (B) Cross-reaction of Western-blotted CM polypeptides with antibodies raised against the holoenzyme (lane 1), subunit I (lanes 2 and 2*), and subunit II (lane 3) of the aa_3-type cytochrome oxidase of *P. denitrificans*. Cross-reacted bands were made visible with goat anti-rabbit horseradish peroxidase conjugate and the 4-chloro-1-naphthol/H_2O_2 color reaction (lanes 1–3) or with ^{125}I-labeled goat anti-rabbit IgG and autoradiography (lane 2*). 30 μg of protein was applied to each lane. The first antibodies were used at dilutions of 1:100 (lane 1), 1:300 (lanes 2 and 2*), and 1:125 (lane 3); the second antibody was used at a dilution of 1:1000 throughout. (C) Marker proteins (Sigma MW-SDS-70L-Kit) stained with Coomassie blue. (b) Immunological cross-reaction of Western-blotted ICM polypeptides with antisera against subunit I (lanes 2), subunit II (lane 3), and the holoenzyme (lane 1) of *P. denitrificans* cytochrome oxidase as described under (a) for the CM. Increasingly purified ICM-0, ICM-I, and ICM-II were used for lanes 2a, 2b, and 2c, respectively. 75 μg of protein was uniformly applied to each lane. Marker proteins are shown on lane 4.

Hoffmann et al., 1980; Seelig et al., 1981; Albracht et al., 1980). Incubation of the membrane preparation with the physiological electron donor NADH or NADPH in the presence of cytochromne *c* and KCN resulted in an almost complete loss of the Cu^{2+} ESR signal (Figure 4c), presumably by the reduction to Cu^{+}. Greater than 90% reduction of the Cu^{2+} ESR signal using the physiological electron donor

Table I: Inhibition and Activation of the Cytochrome Oxidase Activity [nmol of Cytochrome *c* min^{-1} (mg of Protein)$^{-1}$] in 3-Fold-Purified CM and ICM Preparations (CM-III and ICM-III; cf. Figure 2) of *A. nidulans*[a]

additions	final concn	CM	ICM
none		90	2
CO[b]		13	0.3
KCN	1.2 μM	<0.05	<0.05
NaHS	0.1 mM	2.5	<0.05
NaN_3	1.0 mM	3.0	0.1
salicylaldoxime	20 mM	3.0	0.1
NaCl	80 mM	<0.05	<0.05
N_2[c]		<0.05	<0.05
Tween 80[d]	3.5% (w/v)	165	3.5
n-octyl glucoside[d]	0.1% (w/v)	240	5.0

[a] Oxidation of cytochrome *c* was followed by dual-wavelength spectrophotometry at room temperature. Samples contained between 15 and 50 μg of protein/mL and initially 15 μM reduced horse heart cytochrome *c*. [b] Membrane suspensions were flushed with 90% (v/v) CO in air for 10 min. Other inhibitors were added 5 min prior to cytochrome *c*; NaHS-incubated membranes were centrifuged, washed free of sulfide, and finally resuspended in assay medium (10 mM potassium phosphate buffer, pH 7.0). Consistent inhibition patterns were observed with 12 different batches of CM and ICM preparations. [c] Assays conducted anaerobically in Thunberg cuvettes repeatedly evacuated and flushed with oxygen-free nitrogen. [d] Activity in the presence of nonionic detergents was >95% inhibited by 1.2 μM KCN, and no cytochrome *c* oxidation was found in the absence of membranes, thus excluding autoxidations possibly caused by the detergent. By contrast, SDS (0.3%, w/v) elicited high rates of cytochrome *c* oxidation even in the absence of membranes while inhibiting any enzymatic activity (not shown).

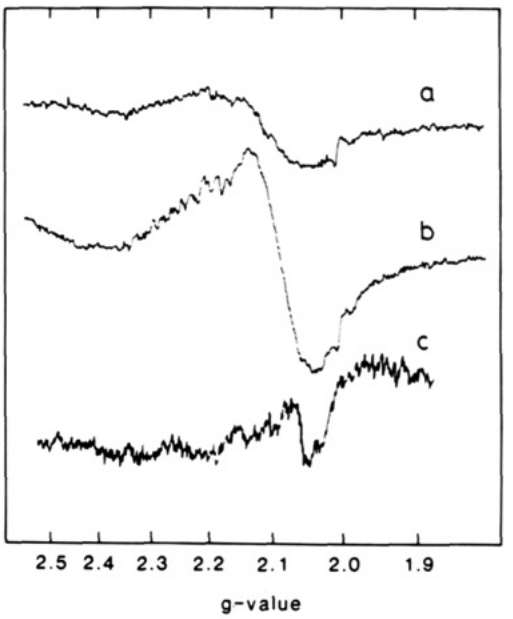

FIGURE 4: Cu^{2+} ESR spectra of purified membrane preparations of *A. nidulans*: (a) ICM; (b) CM; (c) CM plus 2 mM KCN, 0.1 mM horse heart cytochrome *c*, and 30 mM NADH or NADPH. Gain is 3 times that of curves a and b, effects on the ICM signal being the same (not shown). Spectra recorded on submitochondrial particles from beef heart were qualitatively indistinguishable from the spectra shown here [not shown; cf. Albracht et al. (1980) and Fry et al. (1985)]. Conditions of ESR measurements: 20-mW microwave power; 1-mT modulation amplitude; 9.15-MHz frequency; 100-kHz modulation frequency; 60 mT/min scanning rate; 100 K. Approximately 2 mg of protein/mL.

NADH or NADPH in the presence of catalytic amounts of horse heart cytochrome *c* (results not shown) demonstrated the integrity of the complete electron-transport pathway in both CM and ICM (except for the water-soluble endogenous cytochrome *c* lost during purification). By contrast, in the crude membrane preparations reported previously, a certain

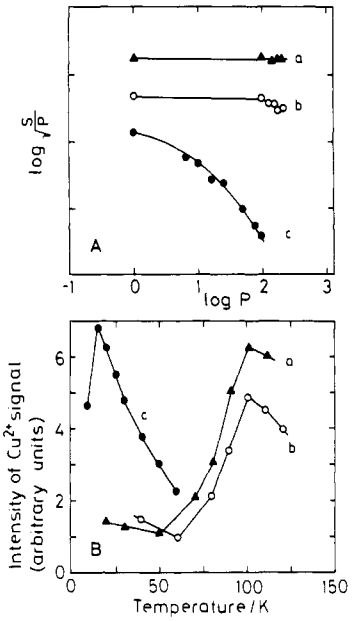

FIGURE 5: Power saturation (A) and temperature dependence (B) of the Cu^{2+} ESR signal in (a) isolated and purified plasma membranes from *A. nidulans*, (b) submitochondrial particles isolated from beef heart (Low & Vallin, 1963), and (c) plastocyanin isolated from *G. violaceus* according to a procedure developed for *Anabaena variabilis* (Lightbody & Krogmann, 1967). Conditions of ESR measurements: (A) 100 K for curves a and b and 15 K for curve c; (B) 20-mW incident microwave power for curves a–c. For other conditions, cf. Figure 4; also cf. Fry et al. (1985).

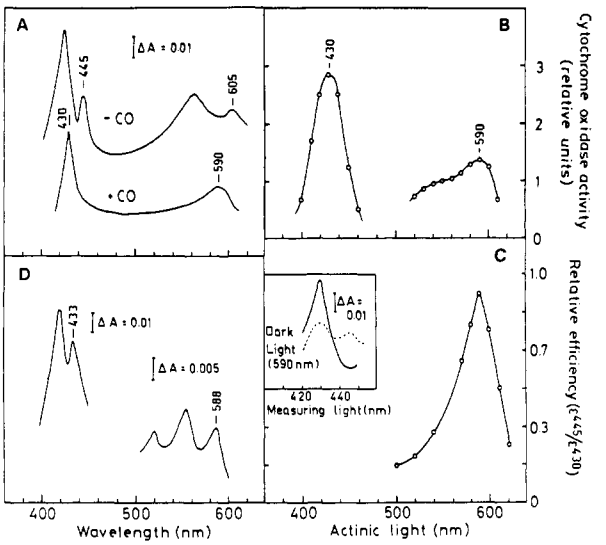

FIGURE 6: Optical spectrophotometry with isolated and purified CM of *A. nidulans*. (A) Ascorbate plus PMS reduced minus H_2O_2-oxidized and ascorbate plus PMS reduced plus CO minus ascorbate plus PMS reduced difference spectra (3.7 mg of protein/mL; 1% *n*-octyl glucoside). (B) Photoaction spectrum of CO-inhibited oxygen uptake by CM preparations (2.7 mg of protein/mL; 0.5% *n*-octyl glucoside) in the presence of 5 mM ascorbate and 0.1 mM horse heart cytochrome *c* (pH 6.9). Oxygen uptake was measured with a Clark-type oxygen electrode (YSI oxygen monitor, Model 53) at 35 °C. Actinic light was provided by an Oriel 1000-W xenon lamp equipped with an Oriel monochromator (half-bandwidth 10 nm). Light intensity was normalized to 20 W/m^2 at each wavelength by use of Kodak Wratten neutral gray filters. Oxygen uptake by the CM preparations was 25–35 nmol min^{-1} (mg of protein)$^{-1}$ without, and about 3 nmol min^{-1} (mg of protein)$^{-1}$ with, CO present. (C) Photodissociation spectrum of the CO-complexed cytochrome aa_3 in the CM preparations (1.4 mg of protein/mL; 0.1% *n*-octyl glucoside). Inset shows the replacement of the CO-complexed γ-peak at 430 nm by the corresponding γ-peak of the CO-free enzyme at 445 nm upon appropriate illumination, the efficiency of which is plotted against the wavelength, maximum efficiency coinciding with the reduced α-peak of the CO-complexed cytochrome at 590 nm. (D) Dithionite-reduced minus 0.1% H_2O_2 oxidized difference spectrum of alkaline pyridine ferrohemochromes prepared by extraction of the CM with 2-butanone/HCl and measured in ether. All spectra were recorded at room temperature on a Shimadzu UV-300 dual-wavelength spectrophotometer equipped with a SAPCOM computer for base-line correction.

fraction of the total ESR-detectable Cu^{2+} was not reducible by physiological electron donors (Fry et al., 1985). This inaccessible Cu^{2+} is attributed to damaged and nonfunctional enzyme (Aasa et al., 1976). The levels of cytochrome oxidase damaged by the present preparation procedures were very low as concluded from a comparison with almost 100% levels of dithionite- or ascorbate/TMPD-reducible Cu^{2+}; quantitatively, the levels of EDTA-resistant and ESR-detectable Cu^{2+} (nmol/mg of protein) were 7.01 and 0.71 in our CM and ICM preparations, respectively (results not shown).

Figure 5 compares microwave power saturation (A) and temperature dependence (B) of the Cu ESR signal at $g = 2.03$ between our purified CM preparations of *A. nidulans* (curve a), submitochondrial particles from beef heart (curve b), and soluble plastocyanin from *Gloeobacter violaceus* (curve c). The results, which are qualitatively very similar to those previously obtained with crude membanes from *Synechococcus* 6311 (Fry et al., 1985), clearly show the close organizational similarity of the tightly bound and EDTA-resistant copper present in beef heart mitochondria [viz., associated with aa_3-type cytochrome oxidase; cf. Beinert et al. (1962), Albracht et al. (1980), and Seelig et al. (1981)] and in our purified *A. nidulans* CM preparations, as opposed to the more loosely bound copper in the soluble Cu protein plastocyanin (Fry et al., 1985).

Optical Spectrophotometry. Ascorbate plus PMS reduced minus H_2O_2-oxidized difference spectra of purified CM (solubilized in 1% octyl glucoside) clearly revealed the γ- and α-bands of an *a*-type cytochrome at 445 and 605 nm which shifted to 430 and 590 nm, respectively, upon treatment of the membranes with CO (Figure 6A). (Peaks around 420 and 563 nm in CO-free samples are due to *b*-type cytochromes which, however, apparently did not react with CO.) When the photoreversibility of CO-inhibited oxygen uptake by CM preparations incubated in the presence of ascorbate and horse heart cytochrome *c* was measured in response to different wavelengths, the effect was most pronounced at 430 and 590 nm (Figure 6B), corresponding to the absorption maxima of the CO-complexed oxidase (cf. Figure 6A). The action spectrum of the photoreversibility of the CO-induced shift of the γ-band of the oxidase from 445 to 430 nm (inset of Figure 6C; also see Figure 6A) gave a maximum at 590 nm (Figure 6C), corresponding to the α-band of the CO-complexed cytochrome aa_3 ("photodissociation spectrum"). Finally, acid-labile heme groups of the CM preparations were extracted with acidic 2-butanone, and alkaline pyridine ferrohemochromes were prepared, extracted into ether, and measured by dithionite-reduced minus H_2O_2-oxidized difference spectrophotometry (Figure 6D); clearly, the pyridine ferrohemochromes of *b*- and *a*-type cytochromes (the latter absorbing at 433 and 588 nm) were present in these extracts.

Influence of Growth Conditions. Experiments aimed at the detection of a possible influence of growth conditions (e.g., medium salinity and time of harvest) on the association of cytochrome oxidase with CM and ICM in *A. nidulans* showed that highest levels of cytochrome oxidase in the CM were measured when the cells were harvested shortly before they entered the stationary growth phase while ICM-bound cytochrome oxidase showed a somewhat opposite correlation (Figure 7A). This might explain why previously neither

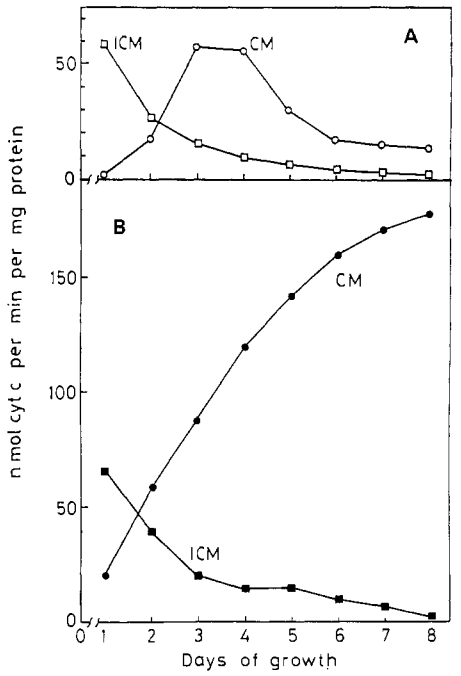

FIGURE 7: Cytochrome oxidase activity in isolated and purified CM and ICM preparations of *A. nidulans* harvested after 1–8 days of growth in normal medium (A; approximately 30 mM NaCl) or in medium supplemented with 0.4 M NaCl (B). Cells grown in the presence of 0.4 M NaCl showed a doubling time about 20% higher than that of control cells and entered the stationary phase only after 8–10 days of growth while control cells did so after 4–5 days of growth (growth curves not shown). It is seen that the effect of elevated NaCl concentration was restricted to the CM. No effect was detected with 0.4 M KCl replacing the NaCl in the growth medium [not shown; cf. Molitor et al. (1986)].

cytochrome oxidase activity (Omata & Murata, 1985) nor *a*-type cytochromes (Omata & Murata, 1984) were found in CM preparations of *A. nidulans* harvested at a cell density of 1 μL/mL. Moreover, Figure 6B shows that elevated levels of Na^+ [but not K^+; controls not shown; cf. Molitor et al. (1986)] in the growth medium drastically enhanced the cytochrome oxidase activity of CM, but not ICM, preparations from *A. nidulans*. This is consistent with the assumption derived from whole cell studies (Fry et al., 1986; Erber et al., 1986) that a CM-bound H^+-translocating cytochrome oxidase provides for a trans-CM proton electrochemical potential gradient that is directly used by a H^+/Na^+ antiporter in this membrane. On the other hand, it appears that cytochrome oxidase synthesis and incorporation into CM and/or ICM are differently regulated in different strains of cyanobacteria [cf. Molitor et al. (1986), Wastyn et al. (1987), Wastyn and Peschek (1988), and Peschek et al. (1988)].

Concluding Remarks

As is shown here for the first time on the grounds of experimental evidence comprising immunology, ESR spectrometry, optical spectrophotometry, and reactivity studies, isolated and purified plasma membranes of a cyanobacterium contain aa_3-type cytochrome oxidase. It is important to realize that the identity of the plasma membranes used in the present study could be unequivocally confirmed by the use of membrane-impermeant protein markers and that the identity of the aa_3-type cytochrome oxidase was established by three independent experimental approaches, viz., immunological cross-reaction, ESR spectrometry of the enzyme-associated copper, and optical spectrophotometry including photoaction and photodissociation spectra. The reason why the results of different investigators are at variance with each other might rest in the different growth conditions (growth phase at the time of harvest, Na^+ concentration in the growth medium, etc.) used for the cells from which the membranes were prepared [H. Wada, G. A. Peschek, and N. Murata, unpublished; also cf. Scherer et al. (1988)]. Attempts to isolate and purify the cyanobacterial aa_3-type cytochrome oxidase by way of affinity chromatography are currently under way in our laboratory [Wastyn et al., 1987, 1988; also cf. Häfele et al. (1988)].

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Registry No. Cyt *c* oxidase, 9001-16-5.

References

Aasa, R., Albracht, S. P. J., Falk, K. E., Lanne, B., & Vanngard, T. (1976) *Biochim. Biophys. Acta 422*, 260–272.

Albracht, S. P. J., Van Versefeld, H. W., Hagen, W. R., & Alkman, M. L. (1980) *Biochim. Biophys. Acta 593*, 173–186.

Aoki, M., & Katoh, S. (1982) *Biochim. Biophys. Acta 682*, 307–314.

Appleby, C. A. (1969) *Biochim. Biophys. Acta 172*, 71–87.

Baccarini-Melandri, A., & Zannoni, D. (1978) *J. Bioenerg. Biomembr. 10*, 109–138.

Beinert, H., Griffith, D. E., Warton, D. C., & Sands, R. H. (1962) *J. Biol. Chem. 237*, 2337–2346.

Berg, H. C. (1969) *Biochim. Biophys. Acta 183*, 65–78.

Bolivar, F., & Backman, K. (1979) *Methods Enzymol. 68*, 245–268.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.

Broda, E. (1975) *The Evolution of the Bioenergetic Processes*, Pergamon Press, Oxford.

Broda, E., & Peschek, G. A. (1979) *J. Theor. Biol. 81*, 201–212.

Douce, R., Block, M. A., Dorne, A. J., & Joyard, J. (1984) *Subcell. Biochem. 10*, 1–84.

Erber, W. W. A., Nitschmann, W. H., Muchl, R., & Peschek, G. A. (1986) *Arch. Biochem. Biophys. 247*, 28–39.

Fry, I. V., Peschek, G. A., Huflejt, M., & Packer, L. (1985) *Biochem. Biophys. Res. Commun. 129*, 109–116.

Fry, I. V., Huflejt, M., Erber, W. W. A., Peschek, G. A., & Packer, L. (1986) *Arch. Biochem. Biophys. 244*, 686–691.

Häfele, U., Scherer, S., & Böger, P. (1988) *Biochim. Biophys. Acta 934*, 186–190.

Hinterstoisser, B., Missbichler, A., Pineau, B., & Peschek, G. A. (1988) *Biochem. Biophys. Res. Commun. 154*, 839–846.

Höchli, L., & Hackenbrock, C. R. (1978) *Biochemistry 17*, 3712–3719.

Hoffmann, B. M., Roberts, J. E., Swanson, M., Speck, S. H., & Margoliash, E. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1452–1456.

Houchins, J. P., & Hind, G. (1984) *Plant Physiol. 76*, 456–460.

Kirk, J. T. O., & Tilney-Bassett, R. A. E. (1978) *The Plastids*, Elsevier Biomedical Press, Amsterdam and New York.

Lightbody, J. J., & Krogmann, D. W. (1967) *Biochim. Biophys. Acta 191*, 508–515.

Low, H., & Vallin, L. (1963) *Biochim. Biophys. Acta 69*, 361–374.

Ludwig, B., & Schatz, G. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 196–200.

Mackinney, G. (1941) *J. Biol. Chem. 140*, 315–322.

Matthijs, H. C. P. (1984) Doctoral Thesis, Vrije Universiteit, Amsterdam.

Molitor, V., & Peschek, G. A. (1986) *FEBS Lett. 195*, 145–150.
Molitor, V., Erber, W., & Peschek, G. A. (1986) *FEBS Lett. 204*, 251–256.
Molitor, V., Trnka, M., & Peschek, G. A. (1987) *Curr. Microbiol. 14*, 263–268.
Morris, J. G. (1975) *Adv. Microb. Physiol. 12*, 169–246.
Morrison, M., & Hopie, S. (1965) *Anal. Biochem. 12*, 77–82.
Omata, T., & Murata, N. (1983) *Plant Cell Physiol. 24*, 1101–1112.
Omata, T., & Murata, N. (1984) *Biochim. Biophys. Acta 766*, 395–402.
Omata, T., & Murata, N. (1985) *Biochim. Biophys. Acta 810*, 354–361.
Peschek, G. A. (1981) *Biochem. Biophys. Acta 635*, 470–475.
Peschek, G. A. (1984) *Plant Physiol. 75*, 968–973.
Peschek, G. A. (1987) in *The Cyanobacteria* (Fay, P., & Van Baalen, C., Eds.) pp 119–161, Elsevier Science, Amsterdam.
Peschek, G. A., & Schmetterer, G. (1982) *Biochem. Biophys. Res. Commun. 108*, 1188–1195.
Peschek, G. A., Schmetterer, G., Lauritsch, G., Nitschmann, W. H., Kienzl, P. F., & Muchl, R. (1982) *Arch. Microbiol. 131*, 261–265.
Peschek, G. A., Molitor, V., Trnka, M., Wastyn, M., & Erber, W. (1988) *Methods Enzymol. 167*, 437–449.
Pineau, B., Dubertret, G., Joyard, J., & Douce, R. (1986) *J. Biol. Chem. 248*, 7215–7222.
Raboy, B., & Padan, E. (1978) *J. Biol. Chem. 253*, 3287–3291.
Reed, R. H., Rowell, P., & Stewart, W. D. P. (1981) *Eur. J. Biochem. 116*, 323–330.
Scherer, S., Almon, H., & Böger, P. (1988) *Photosynth. Res. 15*, 95–114.
Seelig, A., Ludwig, B., Seelig, J., & Schatz, G. (1981) *Biochim. Biophys. Acta 636*, 162–167.
Stanier, R. Y., & Cohen-Bazire, G. (1977) *Annu. Rev. Microbiol. 31*, 225–274.
Tinberg, H. M., Melnick, R. L., Maguire, J., & Packer, L. (1974) *Biochim. Biophys. Acta 345*, 118–128.
Trnka, M., & Peschek, G. A. (1986) *Biochem. Biophys. Res. Commun. 136*, 235–241.
Udenfriend, S., Stein, S., Böhlein, P., Dairman, W., Leimgruber, W., & Weigele, M. (1972) *Science 178*, 871–872.
Wastyn, M., & Peschek, G. A. (1988) *EBEC Rep. 5*, 94.
Wastyn, M., Achatz, A., Trnka, M., & Peschek, G. A. (1987) *Biochem. Biophys. Res. Commun. 149*, 102–111.
Wastyn, M., Achatz, A., Molitor, V., & Peschek, G. A. (1988) *Biochim. Biophys. Acta 935*, 217–224.
Weigele, M., De Bernardo, S. L., Tengi, J. P., & Leimgruber, W. (1972) *J. Am. Chem. Soc. 94*, 5927–5928.

Structural Map of the Dicyclohexylcarbodiimide Site of Chloroplast Coupling Factor Determined by Resonance Energy Transfer†

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ABSTRACT: Fluorescence resonance energy-transfer measurements were made on the membrane-bound chloroplast coupling factor. The distances from the *N,N'*-dicyclohexylcarbodiimide-binding site on the membrane-bound portion of the enzyme (CF_0) to the vesicle surface and to two sulfhydryl sites on the γ-polypeptide were determined. The dicyclohexylcarbodiimide-binding site was labeled with the fluorescent species *N*-cyclohexyl-*N'*-pyrenylcarbodiimide. The vesicle surface was labeled with *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine. Steady-state energy transfer between the fluorescent-labeled enzyme (energy donor) and varying concentrations of the ethanolamine derivative (energy acceptor) indicated that the distance of closest approach between the energy donor and the outer vesicle surface is 16–24 Å. Two specific sites on the γ-polypeptide were reacted with a coumarinylmaleimide derivative; one is a sulfhydryl that can be labeled only on the thylakoids under energized conditions (the "light" site), while the other is the disulfide site that regulates enzymatic activity. Energy-transfer measurements utilizing steady-state fluorescence and fluorescence lifetime methods indicated that the dicyclohexylcarbodiimide site is ~41 Å from the light site and ~50 Å from the γ-disulfide site. These distances are used to extend the current structural model of the chloroplast coupling factor.

The chloroplast ATP synthase catalyzes the photophosphorylation of ADP, coupling ATP synthesis to proton flow down a proton gradient. The enzyme complex consists of an extrinsic portion, chloroplast coupling factor 1 (CF_1),[1] and a membrane-imbedded pore (CF_0). CF_1 is easily solubilized and contains the substrate-binding sites. It is composed of five different polypeptide chains, α, β, γ, δ, and ε, with a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Moroney et al., 1983). CF_0 contains the proton channel and has four different types of polypeptide chains (Pick & Racker, 1979), whose stoichiometry is not precisely known. The smallest of these, a protein

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; CF_0, chloroplast coupling factor 0; DCCD, *N,N'*-dicyclohexylcarbodiimide; NCP, *N*-cyclohexyl-*N'*-(1-pyrenyl)carbodiimide; CPM, *N*-[7-(diethylamino)-4-methylcoumarin-3-yl]maleimide; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; EDTA, ethylenediaminetetraacetic acid.

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