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STUDIES ON THE RED OXIDASE (CYTOCHROME o) OF AZOTOBACTER VINELANDII

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Summary. In an attempt to isolate and to study the electron transport system of Azotobacter vinelandii, we have isolated and purified a membrane-bound cytochrome o. The cytochrome o, purified as a detergent (Triton X-100) and hemoprotein complex, contained 1.6 nmoles heme per mg of protein. Cold-temperature spectrum showed that no other cytochrome was associated with the purified preparation, and electrophoresis revealed that only one type of hemoprotein was obtained. The purified cytochrome o reacted with both carbon monoxide and cyanide readily. Only in the reduced form did it combine with carbon monoxide, whereas the oxidized form reacted with cyanide. An "oxygenated" form of the cytochrome o was demonstrated to be spectrally distinguishable from both the oxidized and the reduced forms.

Cytochrome o was first implicated as a terminal oxidase in bacterial cytochrome systems by Castor and Chance (1). Most bacterial cytochrome oxidases are tightly bound to the intracellular membrane. The consequent difficulty encountered in solubilizing the cytochrome components that are associated with the oxidases has long delayed the understanding of these oxidase systems in bacteria (2,3,4). Jones and Redfearn (5) were the first to solubilize, from the membrane of Azotobacter vinelandii, a red particle that contained an enriched amount of cytochrome o. Jurtshuk et al (6,7) solubilized and purified a TMPD oxidase from the same bacterium, and tentatively identified it as a complex containing c-type cytochrome(s) and cytochrome o. By using a similar procedure, we have isolated and purified the

Abbreviations used: TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine; SDS, sodium dodecylsulfate; DEAE, diethylaminoethyl.

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cytochrome o from Azotobacter vinelandii. Due to its clear reddish appearance in the final preparation, we have named this cytochrome the "red oxidase." The purification and spectral properties of this red oxidase are reported in this communication.

Preparation of electron transport particles. Azotobacter vinelandii strain O was grown in a 200-liter capacity fermenter (New Brunswick Scientific Co.) under conditions previously described (8). Late log phase cells were harvested and washed twice with 0.02M phosphate buffer, pH 7.5. The electron transport particle, designated R_3, was isolated from sonically disrupted cells by differential centrifugation (8).

Solubilization and purification of the red oxidase. A procedure similar to that of Mueller (9) was employed to solubilize the red oxidase from the membrane R_3 fraction. Ammonium sulfate fractionation and DEAE cellulose column chromatography were used to further purify the hemoprotein. The purification procedure and the fractionation scheme are presented in Figure 1 and Table 1, respectively.

Spectral analysis. A Beckman Model 25 spectrophotometer was used for all of the room-temperature spectral studies. A Cary Model 17, equipped with a liquid nitrogen chamber, was used for cold-temperature analysis.

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed in a 5% acrylamide gel, according to the method of Davis (10), using Coomosie brilliant blue for protein staining.

Results. Figure 1 shows the diagramatic procedures for solubilization and purification of the red oxidase from the membrane fraction (R_3) of Azotobacter vinelandii. The ammonium sulfate fraction (27% saturation) contained the majority of cytochrome o in the original membrane fraction, along with minor amounts of c-type cytochrome. This fraction showed some TMPD oxidase activity, possibly due to the presence of a c-type cytochrome that was complexed with cytochrome o. The c-type was removed from this fraction by the DEAE cellulose chromatographic step. The purified red oxidase was eluted as Fraction I from the column by a NaCl gradient (0 - 0.5M) in 0.02M phosphate buffer, pH 7.5, containing 0.1% Triton X-100 (w/v). Table 1 shows a typical fractionation scheme for the purification of the red oxidase. The final preparation represented about a 12-fold purification, in comparison with that of the Triton-treated membrane fraction. The purified hemoprotein contained 1.6 nmoles per mg protein. This value was calculated from the CO:dithionite-reduced minus dithionite-reduced spectrum, using a mM

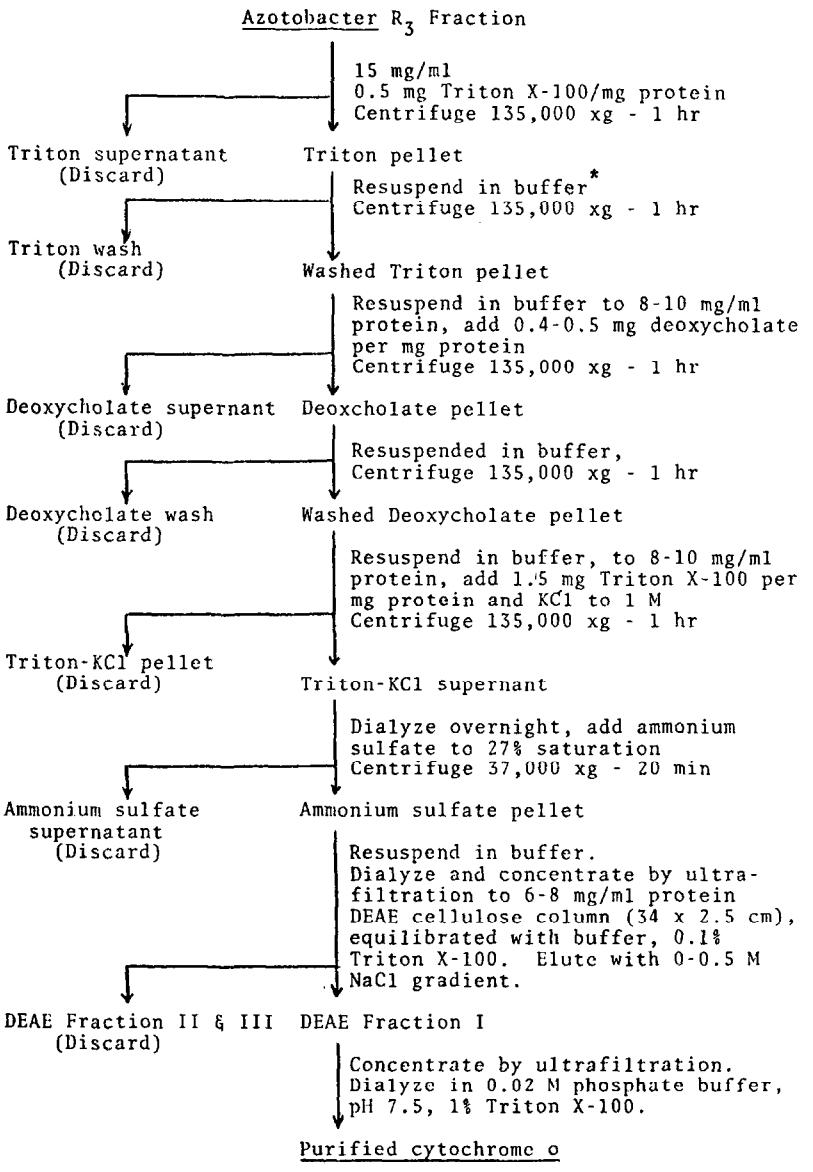


Fig. 1. Scheme for purification of the red oxidase (cytochrome o) from Azotobacter vinelandii electron transport particle, R_3 fraction. All procedures shown were carried out at temperatures below 5° C.

* Buffer used was 0.02M phosphate, pH 7.5, unless otherwise indicated.

extinction coefficient of 170 (11). Electrophoresis of the purified preparation in polyacrylamide gel showed only one protein band, suggesting that a pure cytochrome was obtained. SDS gel electrophoresis of the purified

Table 1. Fractionation scheme for the purification of cytochrome o from the Azotobacter vinelandii membrane.

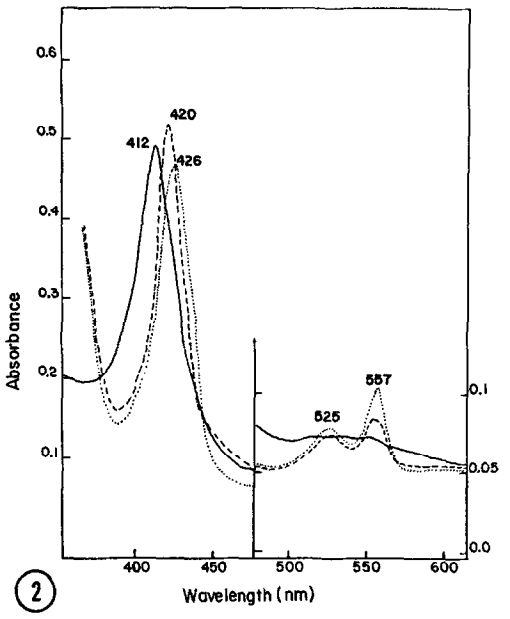
Fraction	Volume (ml)	Total protein (mg)	Cytochrome o concentration* (nmole/mg protein)	Yield (%)
Triton-R_3	176	2640	0.13**	100
Triton-KCl	125	325	0.58	54
Ammonium Sulfate 0-27% saturation	29	116	1.3	44
DEAE Cellulose Column*** Fraction I	20	48	1.6	22

*Calculated as nmole heme per milligram protein, using mM extinction coefficient of 170 (11).
**Estimated value, due to some interference of Triton.
***See Figure 1 for details.

cytochrome has not been accomplished at present.

As shown in Figure 2, the absolute spectrum of oxidized cytochrome o had an absorption maximum at 412 nm; maxima for the reduced form appeared at 426, 525, and 557 nm. Addition of CO to the reduced form shifted the Soret peak to 420 nm and the absorbance increased, while absorbances of both α and β peaks decreased somewhat. The low-temperature spectrum of the purified red oxidase showed the absorption maxima to be at 527 and 555 nm in the visible spectrum (Figure 3). The low-temperature spectrum of the partially purified preparation (ammonium sulfate fraction), as shown in Figure 3 inset, clearly demonstrated the presence of a c-type cytochrome, associated with cytochrome o, which had absorption maxima at 520 and 549 nm. The absorption maxima of the c-type cytochrome were undetectable after column chromatography. The greater purification achieved with this step was also reflected in the slight increase in cytochrome o content, as shown in Table 1.

The oxidized form of the red oxidase was found to react with cyanide quite readily; however, the reduced form showned no reactivity upon spectral



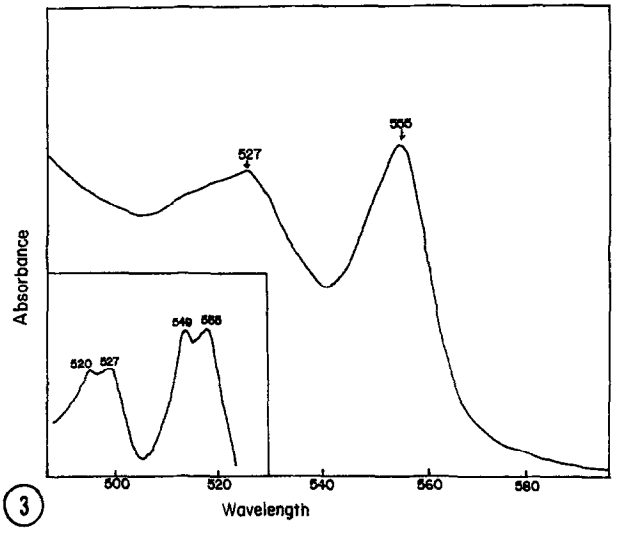
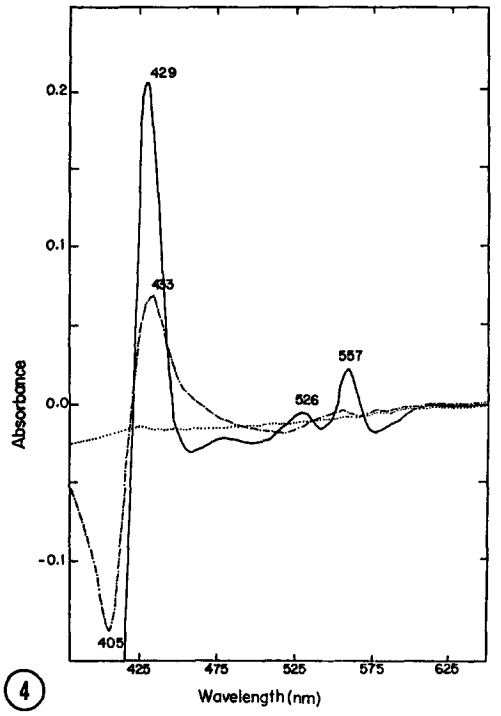


Fig. 2. Absorbance spectra of the purified red oxidase (cytochrome o) from Azotobacter vinelandii membrane fraction. Solid line (———) represents the oxidized cytochrome; dotted line (·····) indicates the reduced form; dashed line (-----) shows the CO-reduced form.

Fig. 3. Low-temperature (77° K) spectrum of the purified red oxidase (cytochrome o) from Azotobacter vinelandii membrane fraction. Inset shows the partially purified preparation, containing some c-type cytochrome, with absorption maxima at 520 and 549 nm.

analysis. The reaction of cyanide with the red oxidase revealed that the spectral alteration was dependent on the final concentration of cyanide. A small amount of dithionite could completely reduce the cytochrome o-cyanide complex, and the spectrum changed to that of reduced cytochrome o. This is shown in Figure 4. After reduction with dithionite, the spectrum had absorption maxima at 429, 526, and 557 nm, which resembled the reduced difference spectrum of the non-cyanide treated preparation.

The red oxidase was found to be highly autooxidizable in a freshly prepared preparation. The steady-state of autooxidation could be achieved in 15 minutes after complete reduction had been carried out in a preparation containing 0.5 mg/ml hemoprotein. A so-called "oxygenated" form was obtained by reducing the cytochrome o with a small amount of dithionite, and subsequent-



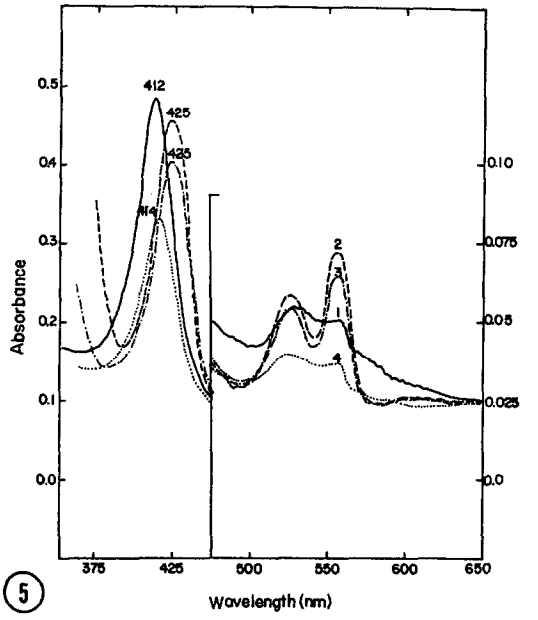


Fig. 4. Cyanide and reduced difference spectra of the red oxidase (cytochrome o). Dotted line (·····) represents the base line (oxidized minus oxidized); dashed line (-·-·-) shows the cyanide plus oxidized spectrum; solid line (———) represents cyanide plus reduced spectrum.

Fig. 5. Time-course spectra of the "oxygenation" of cytochrome o purified from Azotobacter vinelandii. Curve 1 (———) is the oxidized preparation; curve 2 (-----) the reduced form at 1 minute after dithionite reduction; curve 3 (-·-·-) the reduced form after 10 minutes; curve 4 (·····) the "oxygenated" form.

ly aerating the reduced form for about 30 seconds. Alternatively, this "oxygenated" form could also be approached gradually by exposing the reduced form to the air after a brief shaking. As shown in Figure 5, the untreated preparation had its maximum at 412 nm, and the reduced form at 425 nm in the Soret region. The effect of "oxygenation" shifted the reduced peak back to 414 nm, with a substantial decrease in absorbance. Thus, the form that had a Soret maximum at 414 nm is considered as an "oxygenated" form of the cytochrome o. Table 2 shows that the spectral shift during "oxygenation" was a function of time after reduction. Interestingly, the untreated preparation

Table 2. Spectral changes during "oxygenation" of the partially purified cytochrome o upon shaking and standing in the air, after reduction was carried out with dithionite. Sample contained 0.5 mg hemoprotein per ml.

	Time elapsed after reduction (minutes)				
	1	10	20	35	untreated
Soret band					
wavelength	423	423	414	414	412
absorbance	0.453	0.402	0.333	0.333	0.481
α band					
absorbance	0.048	0.041	0.016	0.013	0.026

had much more absorbance, in both visible and Soret regions, in comparison with that of the "oxygenated" form, possibly indicating that a partially reduced cytochrome o was obtained after purification. This "oxygenated" form was highly stable, and it reacted with CO.

Discussion. The spectral changes that resulted from treating the reduced red oxidase with CO have partially confirmed the early work of Castor and Chance on the CO action spectrum studies with Azotobacter oxidase systems (1). The cyanide spectrum studies have also supported previous observations that cytochrome o, in Azotobacter vinelandii, is a cyanide-sensitive oxidase. This is, in part, substantiated by the evidence showing that TMPD oxidase, a complex composed of cytochromes c and o, is very sensitive to the inhibition with cyanide (6,7,9). Unlike the soluble cytochrome o that has been purified from Vitreocilla (12), no NADH-cytochrome o reductase activity could be detected in association with the red oxidase. Throughout the purification process, it was evident that cytochrome o was tightly bound to the membrane and was associated with a c-type cytochrome. No cytochrome a_1 was found to be solubilized together with cytochrome o or with cytochrome c, suggesting that, in the membrane fraction of Azotobacter vinelandii, cytochrome a_1 and cytochrome o may not be associated, as once suggested (13).

An "oxygenated" form of the red oxidase, obtained by aerating the reduced form, was demonstrated to be spectrally different from both the reduced and the oxidized (or untreated) forms. The stability and its reactivity with CO suggest a possible functional role in the oxidase reaction mechanism, analogous to that of the mammalian oxidase. Further studies to determine the significance of the "oxygenated" red oxidase are now under way.

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