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Cytochrome *c* Oxidation by the Electron Transport Fraction of *Azotobacter vinelandii*

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The spectrophotometric oxidation of horse heart ferrocytochrome *c* was examined by use of the particulate electron transport fraction (R_3) of *Azotobacter vinelandii* strain O. Unlike cytochrome *c*, purified preparations of native *Azotobacter* cytochromes $c_4 + c_5$ were oxidized only slowly by the electron transport fraction. The oxidation of mammalian cytochrome *c* proceeded at an appreciable rate and displayed "apparent" first-order kinetics at a *p*H optimum of 9.0 with tris(hydroxymethyl)aminomethane-chloride buffer. The calculated V_{max} value was 0.22 μmole of cytochrome *c* oxidized per min per mg of protein (25 C) and a K_m value for cytochrome *c* of 2.3×10^{-5} M was obtained. Ferricytochrome *c* was a "strict" competitive inhibitor for this oxidation. Cytochrome *c* oxidation by the *Azotobacter* electron transport system was markedly sensitive to cyanide, azide, and hydroxylamine, although carbon monoxide inhibition could not be demonstrated. It was sensitive also to high concentrations of phosphate, ethylenediaminetetraacetate, and some metal cations. "Aging" or prolonged storage of the *Azotobacter* R_3 fraction, at 4 C for 10 days, resulted in a threefold increase in specific activity. The cytochrome *c* peroxidase type of reaction did not occur with the R_3 electron transport fraction.

It has been shown previously that mammalian cytochrome *c* is oxidized by cell-free extracts of *Azotobacter vinelandii* (9). A continuation of these studies has revealed that horse heart cytochrome *c* was oxidized by the electron transport fraction of *A. vinelandii* strain O at a rate at least 10-fold greater than that observed for the isolated, native cytochromes c_4 and c_5 of *A. vinelandii*. These rates were still low when compared to those values obtained for cytochrome *c* oxidation with mammalian mitochondria. Therefore, the oxidation of cytochrome *c* by the *Azotobacter* electron transport fraction was examined to define those kinetic parameters that would lead to the development of a spectrophotometric assay. This could then facilitate in the isolation and purification of that portion of the terminal oxidase enzyme complex responsible for cytochrome *c* oxidation. In *Azotobacter* spp., it has been postulated that the terminal oxidase function is carried out by cytochromes a_1, a_2, and *O*.

MATERIALS AND METHODS

Source of materials. Horse heart cytochrome *c* (type II), ethylenediaminetetraacetic acid (EDTA), adenosine triphosphate (ATP), bovine liver catalase, and Trizma base or tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma Chemical Co., St. Louis, Mo. Hydroxylamine hydrochloride and sodium azide were from Matheson Co., Inc., East Rutherford, N.J. Potassium ferricyanide and glycine were obtained from Fisher Scientific Co., Pittsburgh, Pa. Palladium (10%) on asbestos was from K & K Laboratories, Jamaica, N.Y., and potassium cyanide from J. T. Baker Chemical Co., North Phillipsburg, N.J.

Preparation of c-type cytochrome substrates. Mammalian cytochrome *c* was prepared routinely with deionized water (10 mg/ml) and was stored at −15 C. Chemical reduction was carried out with use of hydrogen and palladium according to Smith's method (11).

Azotobacter cytochromes $c_4 + c_5$ were prepared routinely with deionized water (10 mg/ml) and were stored at −15 C. Chemical reduction was carried out by use of hydrogen and palladium according to Smith's method (11).

Azotobacter cytochromes $c_4 + c_5$ were obtained by the procedure described by Tissieres (12), with the following modifications. Only 2 ml of a 25% (w/v) solution of basic lead acetate was added per 100 ml of cytochrome $c_4 + c_5$ solution after the butyl alcohol extraction step. This was done to avoid excess precipitation of protein. After treatment with lead acetate, solid ammonium sulfate was used for precipitation of proteins. The *Azotobacter* cytochromes $c_4 + c_5$ precip-

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itated at both the 60 to 70 and 70 to 80% ammonium sulfate saturation levels. Reduction of cytochrome $c_4 + c_5$ for assays was carried out by use of hydrogen and palladium. Analyses of these two purified fractions revealed a sharp absorbance peak at 551 mμ, which suggested that this preparation consisted predominantly of cytochrome c_4 with a small amount of cytochrome c_5.

Protein determinations. Protein concentrations of all cytochrome *c* oxidase preparations were determined by the Biuret method of Gornall et al. (6).

Preparation of Azotobacter R_3 electron transport fraction. A. vinelandii strain O cells were grown on a modified Burk's nitrogen-free liquid medium with 1% sodium acetate as the sole carbon source and atmospheric nitrogen as the sole nitrogen source. The cells were harvested, standardized turbidimetrically, and exposed to intermittent sonic oscillation at 4 C. The particulate electron transport fraction (designated *Azotobacter* R_3) was obtained by differential centrifugation, and it represents the fraction that was not sedimented at 37,000 × *g* for 20 min but was sedimented at 144,000 × *g* for 120 min (8). The R_3 fraction appeared as a dark reddish brown translucent pellet, and of all of the fractions it contained the highest reduced nicotinamide adenine dinucleotide ($NADH_2$), succinate and tetramethyl-*p*-phenylenediamine oxidase activities (8). The R_3 fraction (Fig. 1) contained high concentrations of cytochromes and flavoprotein components, and its spectral characteristics were typical for those attributed to the electron transport particle or fraction of *A. vinelandii*. Figure 1 shows a difference spectrum of the R_3 fraction (dithionite reduced minus oxidized) showing prominent oxidoreductive changes at 629, 600, 561, 553, 531, 524, and 457 which correspond to the α and β regions of cytochromes a_2, a_1, b_1, c_4 and c_5, and flavoprotein.

Preparation of beef kidney mitochondria. Mitochondria were prepared from beef kidney cortical tissue by the procedure described by Ishikawa, Oliver, and Reed (7). All mitochondrial preparations were extensively washed in 0.25 M sucrose and, prior to use in assays, all dilutions were made in 0.02 M phosphate buffer, *p*H 7.5.

Assay for cytochrome c oxidase. Assays for the *Azotobacter* cytochrome *c* oxidase were carried out at 25 C by measuring the oxidation of reduced cytochrome *c* at 550 mμ in a Cary model 14 recording spectrophotometer. The final reaction mixture of 1.0 ml contained 50 μmoles of Tris-chloride buffer, *p*H 9.0, 20 to 60 mμmoles of reduced cytochrome *c*, the R_3 fraction, and deionized water. The reaction was initiated by the addition of enzyme, after temperature equilibration, and the concentration of oxidized cytochrome *c* was estimated at completion of the reaction. This was done by estimating optical density change at 550 mμ after the addition of 0.02 ml of a saturated solution of $K_3Fe(CN)_6$ to the assay cuvette (11). Specific activity was calculated by measuring the initial rate of reaction, which represented the extrapolated value obtained for the first 20-sec interval. All specific activities are reported as micromoles of cytochrome *c* oxidized per min per mg (at 25 C). For all calculations the extinction coefficient used for cytochrome *c* was 18.5 $cm^{-1} \times mM^{-1}$ (10) and that for *Azotobacter* cytochromes $c_4 + c_5$ was 23.8 $cm^{-1} \times mM^{-1}$ (12).

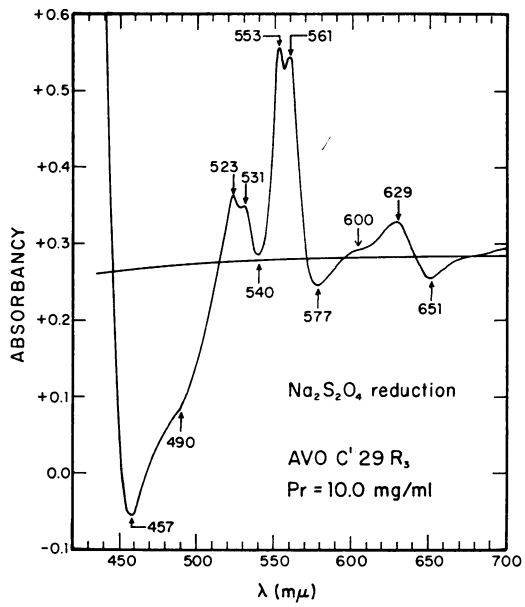

FIG. 1. *Difference spectrum (reduced minus oxidized) of the Azotobacter R_3 fraction clarified by Triton X-100. Chemical reduction was carried out by the addition of sodium hydrosulfite. This fraction exhibited the highest specific activity for cytochrome c oxidation, in addition to $NADH_2$, succinate, tetramethyl-p-phenylenediamine, and p-phenylenediamine oxidase activities (8).*

Assay for cytochrome $c_4 + c_5$ oxidase. The enzymatic oxidation of cytochromes $c_4 + c_5$ was estimated in the identical manner described above, except that optical density changes were followed at 551 mμ. Lower concentration levels of the *Azotobacter* cytochromes $c_4 + c_5$ were used in the assays.

RESULTS

Oxidation of cytochromes $c_4 + c_5$ and cytochrome c. Both mammalian (horse heart) cytochrome *c* and *Azotobacter* cytochromes $c_4 + c_5$ were oxidized by both the *Azotobacter* R_3 fraction and beef kidney mitochondria (Table 1). When cytochromes $c_4 + c_5$ were used as the electron donor, *p*H 7.5, with phosphate buffer, the *Azotobacter* R_3 fraction exhibited a specific activity of only 0.002 μmoles of cytochrome $c_4 + c_5$ oxidized per min per mg of protein. It is quite possible that residual lead may still have been present in the isolated cytochrome $c_4 + c_5$ preparation, and this would then account for the low cytochrome $c_4 + c_5$ oxidase rate. No cytochrome $c_4 + c_5$ oxidation rate was detected when the assay for the *Azotobacter* system was carried out in Tris buffer, *p*H 9.0. Beef kidney mitochondria,

TABLE 1. *Comparative study on the rates of oxidation of cytochromes $c_4 + c_5$ and cytochrome c by the Azotobacter R_3 fraction and beef kidney mitochondria*

Electron donor	Assay buffer	pH	Specific activity[a]	
			Azotobacter R_3	Mitochondria
Cytochrome $c_4 + c_5$ (6 μM)	PO_4	7.5	0.002	0.015
	Tris	9.0	0	—
Cytochrome *c* (23 μM)	PO_4	7.5	0.023	1.200
	Tris	9.0	0.167	0.400

[a] Micromoles of cytochrome $c_4 + c_5$ or cytochrome *c* oxidized per min per mg of protein (at 25 C) with the indicated concentrations of cytochrome $c_4 + c_5$ or cytochrome *c*.

however, were able to oxidize cytochromes $c_4 + c_5$ at a rate of 7.5 times faster with a specific activity of 0.015. Therefore, the *Azotobacter* electron transport fraction oxidized its own natural acceptor at a slow rate, which represents only 11% of the velocity attained with beef kidney mitochondria. Furthermore, in the presence of phosphate buffer, *p*H 7.5, the *Azotobacter* R_3 fraction oxidized horse heart cytochrome *c* at a rate 11.5 times faster than the *Azotobacter* cytochrome $c_4 + c_5$ (Table 1). The higher cytochrome *c* concentration would account for only a twofold increase in the oxidation rate over cytochromes $c_4 + c_5$. In addition, it was noted that cytochromes $c_4 + c_5$ were incapable of oxidation by the R_3 fraction, *p*H 9.0, in Tris-chloride buffer, even at the low rate obtained at *p*H 7.5 with phosphate buffer. However, cytochrome *c* oxidation was markedly greater in Tris-chloride buffer, *p*H 9.0, and was 7.3 times greater than the comparable rate obtained at *p*H 7.5 in phosphate. In contrast to the *Azotobacter* system, the increase in *p*H inhibited the mitochondrial cytochrome *c* oxidation by threefold. The ability of the *Azotobacter* electron transport fraction to oxidize mammalian cytochrome *c*, in alkaline *p*H at a rate approximately 84 times greater than the oxidation of its native cytochrome $c_4 + c_5$, warranted the reinvestigation of the problem of cytochrome *c* oxidation by the *Azotobacter* system.

Effect of pH on cytochrome c oxidation. The effect of *p*H on the oxidation of cytochrome *c* by the *Azotobacter* R_3 electron transport fraction is shown in Fig. 2. The term "dialyzed enzyme" refers to an *Azotobacter* R_3 fraction which was prepared in 0.02 M phosphate buffer, *p*H 7.5, but from which the phosphate ion had been removed by prolonged dialysis against 0.02 M Tris-chloride buffer, *p*H 8.0. "Tris enzyme" refers to an *Azotobacter* R_3 fraction which was prepared, suspended, and assayed solely in Tris-chloride buffer. (Every attempt was made to prevent the exposure of the R_3 fraction to phosphate ion.) Activity for cytochrome *c* oxidation was maximal when the Tris enzyme was assayed in Tris-chloride buffer, *p*H 9.0. Above *p*H 9, the activity declined rapidly until at *p*H 10.5 only negligible oxidation occurred. Removal of the phosphate ion from an *Azotobacter* R_3 fraction (dialyzed enzyme) produced an altered R_3 enzyme preparation which exhibited high cytochrome *c* oxidase activity when assayed also in Tris-chloride buffer. Again, the highest specific activity was observed at *p*H 9.0. When the dialyzed enzyme was assayed in 0.05 M phosphate buffer, its specific activity was substantially lower when compared to the rate obtained in Tris-chloride buffer, even within the same *p*H ranges. From this study it was possible to conclude that phosphate ion was inhibitory for cytochrome *c* oxidation, and that this inhibitory effect was more apparent at the neutral or alkaline *p*H range (7.0 to 8.0) rather than at acidic *p*H (6.0 to 6.5).

Inhibition by phosphate ion. The oxidation of

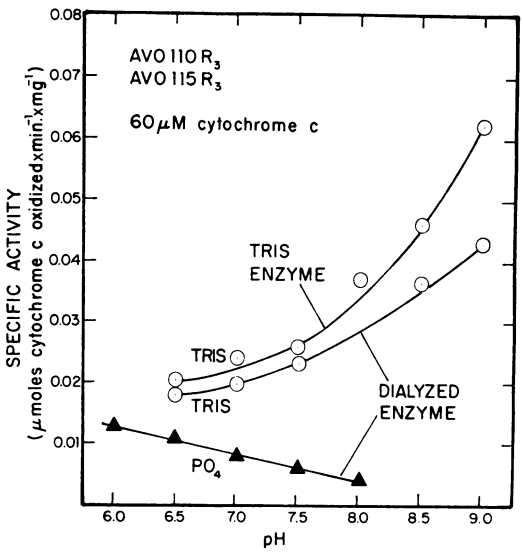

FIG. 2. *Effect of pH on cytochrome c oxidation by the Azotobacter R_3 electron transport fraction. The term dialyzed enzyme refers to an Azotobacter R_3 fraction which was prepared in 0.02 M phosphate buffer but from which the phosphate had been removed by prolonged dialysis against 0.02 M Tris buffer, pH 8.0. Tris enzyme refers to an Azotobacter R_3 fraction which was prepared and suspended in Tris-chloride buffer.*

cytochrome *c* by the *Azotobacter* electron transport particle was inhibited by the presence of phosphate ion. The degree of inhibition was proportional to the phosphate ion concentration in the assay cuvette. At a phosphate ion concencentration of 5×10^{-2} M (a concentration level that would be used routinely in most assays) 83% inhibition of cytochrome *c* oxidation occurred. At 5×10^{-3} M phosphate, 36% inhibition occurred. No inhibition occurred at a concentration level of 10^{-3} M phosphate. Preliminary studies also indicated that compounds, such as ATP (10^{-3} M), inhibited cytochrome *c* oxidation by the *Azotobacter* R_3 enzyme fraction. It is unlikely that the phosphate liberated by an adenosine triphosphatase would have been responsible for this inhibition because the final concentration of ATP used was 10^{-3} M.

Time course and kinetics of cytochrome c oxidation. Figure 3 (A and B) shows the typical time course of reaction for cytochrome *c* oxidation by the *Azotobacter* R_3 fraction. The oxidation was carried out in Tris-chloride buffer, *p*H 9.0, at a cytochrome *c* concentration of 23 μM. To oxidize about 75% of the cytochrome *c* present, 4 min were required. The specific activity was 0.195 μmole of cytochrome *c* oxidized per min per mg of protein (at 25 C). Figure 3B represents a semilog plot of the data (Fig. 3A). The rate constant for cytochrome *c* oxidation obtained for the *Azotobacter* R_3 fraction, calculated from the slope of the straight line according to Smith's method (11), was found to be 0.0032 sec^{-1}. It can be concluded that oxidation of mammalian cytochrome *c* by the *Azotobacter* R_3 fraction appears to follow first-order kinetics. This is the type of reaction kinetics commonly observed with the oxidation of cytochrome *c* by mammalian mitochondria.

Effect of ferrocytochrome c on kinetics of cytochrome c oxidation. Figure 4 illustrates the effect of cytochrome *c* concentration on the reaction velocity of the *Azotobacter* R_3 cytochrome *c* oxidase. Specific activity is plotted as a function of cytochrome *c* concentration (Fig. 4A). The enzymatic cytochrome *c* oxidation of the *Azotobacter* R_3 fraction was proportional to cytochrome *c* concentration until the 60 μM concentration level was reached. A sharp decline in specific activity occurred at concentration levels exceeding the 60 μM level which is characteristic of substrate inhibition. At a concentration level of 110 μM, the specific activity of the R_3 fraction was reduced by 50%, whereas at the 140 μM cytochrome *c* level negligible cytochrome *c* oxidase activity was observed. Figure 4B shows the Lineweaver-Burk analysis of the data (Fig. 4A). A curve typical of substrate inhibition was observed (4). Calculation of the V_{max} gave a value of 0.22 μmole of cytochrome *c* oxidized per min per mg of protein, and a K_m value of 2.3×10^{-5} M cytochrome *c*.

Effect of ferricytochrome c on kinetics of cytochrome c oxidation. Figure 5 shows the reaction kinetics obtained when oxidized cytochrome *c* was added to the assay. The lower straight line of this double reciprocal analysis represents the control substrate titration curve at ferrocytochrome *c* concentration levels that ranged from

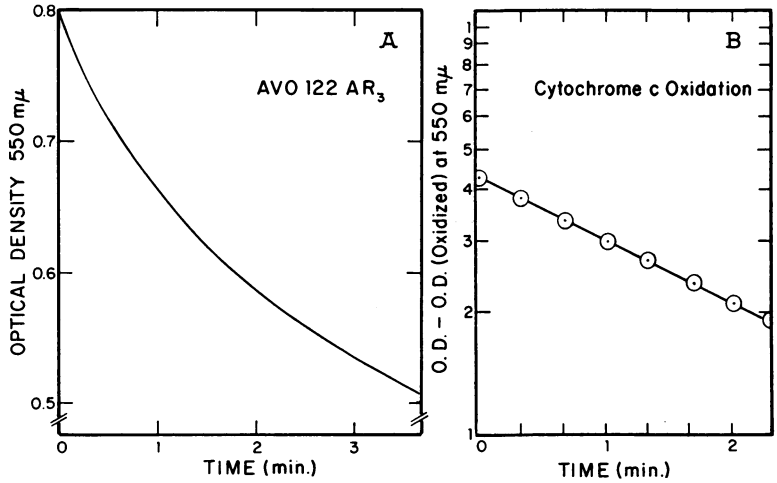

FIG. 3. *Typical time course reaction for cytochrome c oxidation by the Azotobacter R_3 fraction. The final protein concentration was 50 μg per ml, and the concentration of cytochrome c was 23 μM. Specific activity (calculated from the initial rate of reaction) was 0.195 μmole of cytochrome c oxidized per min mg of protein. The first-order rate constant was $k = 0.0032$ sec^{-1}.*

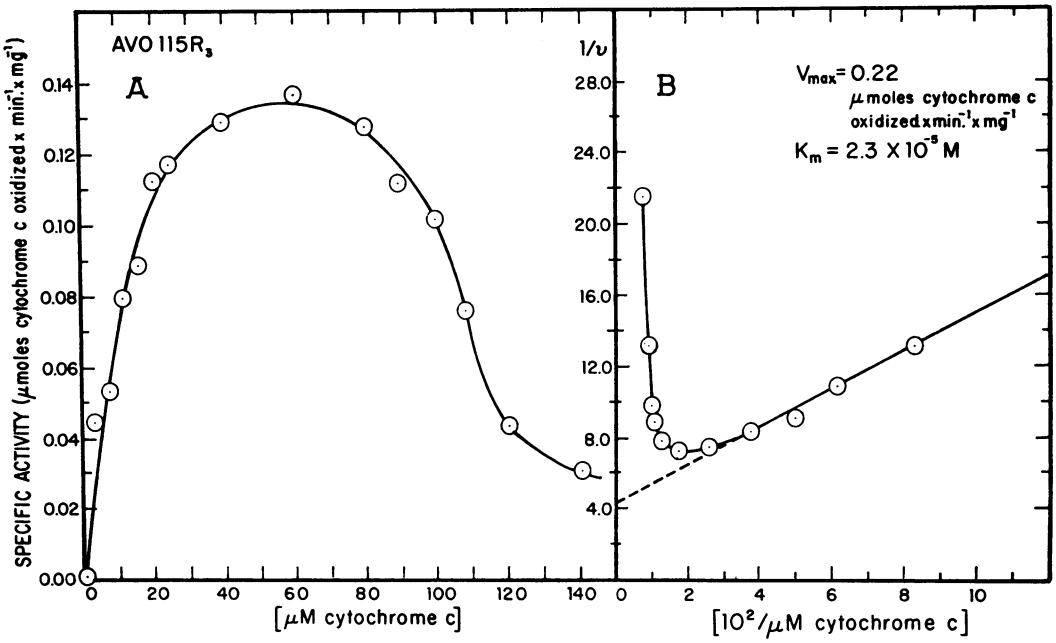

FIG. 4. *Effect of cytochrome c concentration on cytochrome c oxidation by the Azotobacter R_3 fraction. The final concentration of R_3 fraction used was 64 μg of protein per ml.*

20 to 80 μM. The upper straight line represents the titration data obtained in the identical manner, except that 16 μM oxidized cytochrome *c* was present in the reaction mixture. Classical competitive inhibition by the ferricytochrome *c* was obtained. In this study, the calculated K_i value for ferricytochrome *c* was 1.6×10^{-5} M, which was approximately equal to the K_m value of 1.5×10^{-5} M for ferrocytochrome *c*.

Effect of "aging". It was noted that although the same *Azotobacter* R_3 enzyme preparation was used in both assay series, different kinetic parameters were obtained (Fig. 4B and 5). The only difference between these two series of experiments were the days at which the assays were performed. Differences in kinetic parameters were noticeable for the K_m values given for cytochrome *c* oxidation (2.3×10^{-5} M for Fig. 4 and 1.5×10^{-5} M for Fig. 5). A similar type effect was also observed for the V_{max} values, although the differences were much smaller in magnitude. This effect could be repeated consistently and was caused by aging or the prolonged incubation of the *Azotobacter* R_3 fraction at 4 C. Further studies on the *Azotobacter* R_3 fraction revealed that not only was the cytochrome *c* oxidase activity stable during prolonged incubation at 4 C but also with each subsequent day of aging (at 4 C) an increase in specific activity occurred. An overall 3.5-fold increase in specific activity could be obtained by

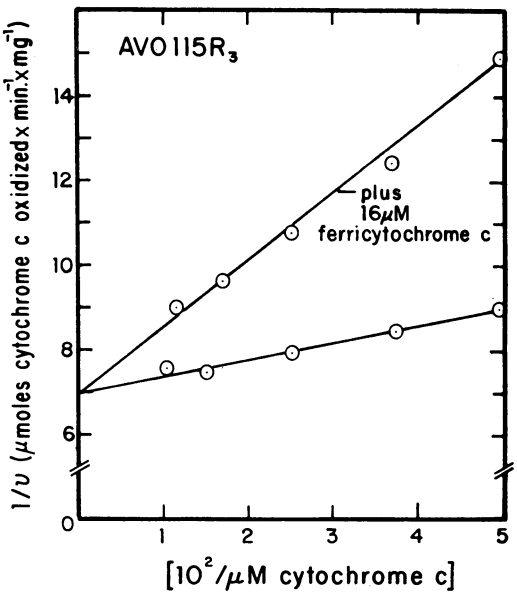

FIG. 5. *Effect of ferricytochrome c on ferrocytochrome c oxidation by the Azotobacter R_3 fraction. A constant amount of oxidized cytochrome c (16 μM) was added to various concentrations of ferrocytochrome c. The final concentration of R_3 fraction was 64 μg of protein per ml. K_m (ferrocytochrome c) = 15 μM. K_i (ferricytochrome c) = 16 μM.*

storing the R_3 fraction for 7 days at 4 C. The aging phenomenon explained many of the discrepancies in specific activity and other kinetic parameters observed throughout this study. That is, the apparent velocities or specific activities were dependent both on the day of assay and the conditions of storage of the *Azotobacter* R_3 fraction.

Effect of cytochrome c oxidase inhibitors. Figure 6 illustrates the effect of potassium cyanide, sodium azide, and hydroxylamine on cytochrome *c* oxidation by the *Azotobacter* R_3 fraction. The percentage of inhibition of cytochrome *c* oxidation is plotted as a function of the final molar concentration of inhibitor employed in the assay. Although the inhibitor data (Fig. 6) were obtained with an *Azotobacter* R_3 fraction assayed in phosphate buffer, almost identical inhibitor values were obtained at specific but comparable inhibitor concentrations with an R_3 fraction assayed in Tris buffer.

Cytochrome *c* oxidation by the *Azotobacter* R_3 fraction was extremely sensitive to cyanide, 50% inhibition occurring at 9×10^{-7} M. The *Azotobacter* cytochrome *c* oxidase system was apparently also very sensitive to both azide and hydroxylamine, 50% inhibition occurring at 4.5×10^{-6} and 1.5×10^{-4} M, respectively. Repeated attempts to demonstrate carbon monoxide inhibition of cytochrome *c* oxidation by the *Azotobacter* R_3 fraction were unsuccessful.

Inhibition by EDTA and metal cations. When the *Azotobacter* R_3 fraction was assayed for cytochrome *c* oxidase activity in the presence of EDTA, substantial inhibition of enzymatic activity was observed. At 5×10^{-5} M EDTA, the *Azotobacter* cytochrome *c* oxidase activity was inhibited 78%. Since EDTA exhibited an inhibitory effect, the effect of metal ions on cytochrome *c* oxidation by the *Azotobacter* electron transport fraction was examined.

Metal ions also were found to be quite inhibitory for *Azotobacter* cytochrome *c* oxidation. At 10^{-3} M magnesium chloride concentration, the *Azotobacter* cytochrome *c* oxidase activity was inhibited 73%. Manganese, strontium, barium, and calcium were potent inhibitors at this concentration level. Neither copper nor aluminum inhibited the cytochrome *c* oxidation, and the effect of iron could not be tested because addition of this metal precipitated the contents of the assay cuvette. Apparently, inhibition by metals of cytochrome *c* oxidation by the *Azotobacter* electron transport fraction is nonspecific. Mitochondrial cytochrome *c* oxidation was neither inhibited by EDTA nor metal ions.

Stability of cytochomre c oxidase activity. The cytochrome *c* oxidase of the *Azotobacter* R_3 electron transport fraction appeared markedly

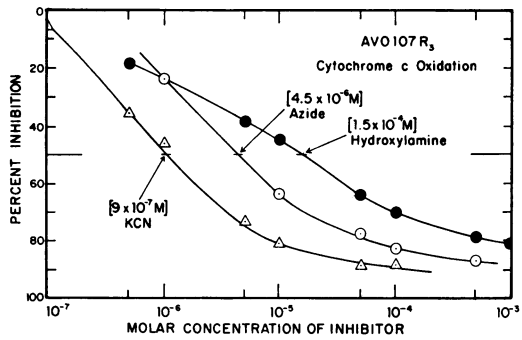

FIG. 6. *Effect of cytochrome c oxidase inhibitors on cytochrome c oxidation by the Azotobacter R_3 fraction. The assay was carried out in 0.02 M phosphate buffer, pH 7.5, with 60 μM cytochrome c. Final concentration of the R_3 fraction was 510 μg of protein per ml. The figures in brackets indicate the molar concentration of the inhibitor at which a 50% inhibition value was estimated by interpolation.*

stable under a variety of conditions. Substantial activity was retained even after several months of storage at −15 C (at protein concentrations of 15 mg per ml). Physical aggregation of the *Azotobacter* R_3 fraction, which resulted from prolonged storage at this temperature, was altered by brief exposure to sonic oscillation without loss of activity. However, for routine use in spectrophotometric assays, it was desirable to use a freshly prepared *Azotobacter* R_3 electron transport fraction which had a high translucent appearance. The *Azotobacter* R_3 cytochrome *c* oxidase was extremely stable to dialysis against 0.02 M Tris-chloride, *p*H 8.0 and *p*H 9.0, for extended periods of time. The *Azotobacter* cytochrome *c* oxidase activity was heat-sensitive, and exposure of the *Azotobacter* R_3 fraction to 70 C for 5 min resulted in complete loss of activity.

DISCUSSION

Initial rates of cytochrome *c* oxidation have been found to be strictly proportional to enzyme concentration. The kinetic parameters obtained from the Lineweaver-Burk analysis agree quite readily with those reported for both the mammalian and microbial systems (9, 13, 17). It was also observed that the oxidation of cytochrome *c* displays "apparent" first-order reaction kinetics. When the data of a typical time course reaction were subjected to analysis by the classical log treatment, a straight line was obtained, the slope of which expressed the first-order rate constant (Fig. 3).

Yonetani and Ray (17), who have derived the various rate equations which predict the time course kinetics (for mammalian cytochrome *c*

oxidation), demonstrated that when the K_m (ferrocytochrome *c*) value equals the K_i (ferricytochrome *c*) value, the time course of the reaction was "apparent" first order. This appears to be the case with *Azotobacter* cytochrome *c* oxidation. The K_m value of ferrocytochrome *c* is 1.5×10^{-5} M and approximately equal to the K_i value of 1.6×10^{-5} M of ferricytochrome *c* (Fig. 5).

The oxidation of mammalian cytochrome *c* by the *Azotobacter* R_3 system showed some properties not commonly associated with mammalian cytochrome *c* oxidation. Mammalian cytochrome *c* oxidation is optimal at neutral *p*H values (15), whereas the *Azotobacter* R_3 cytochrome *c* oxidase exhibited a high *p*H optimum (*p*H 9.0). Another microbial cytochrome *c* oxidase from *Pseudomonas aeruginosa* has been reported to have a *p*H optimum in Tris-chloride buffer from *p*H 8.0 to 8.5 (2). The *Azotobacter* R_3 cytochrome *c* oxidase activity was also inhibited by phosphate ion. This finding agrees with the investigations by Layne and Nason (9), who reported on phosphate inhibition of the cytochrome *c* oxidase, and Wilson and Wilson (14), who reported on succinic oxidase activities with *Azotobacter* cell-free extracts. Our studies showed that the inhibition of the cytochrome *c* oxidase activity by phosphate is reversible and is accomplished by the removal of phosphate ion by dialysis.

EDTA also inhibited cytochrome *c* oxidation by the *Azotobacter* R_3 fraction. Duncan (5) found recently that yeast cytochrome *c* oxidase is strongly inhibited by 0.001 M EDTA and stated that a nonheme iron or copper may be involved in the catalysis. Also, preliminary studies suggest that many divalent cations appear to inhibit the terminal *c* oxidase activity in the R_3 fraction. The *Pseudomonas* cytochrome *c* oxidase also was reported to be sensitive to certain metal ions (2). Both EDTA and metal sensitivity are unusual for a cytochrome *c* oxidase, and further clarification of these effects in terms of mechanism are necessary.

Another unusual feature noted for the *Azotobacter* R_3 cytochrome *c* oxidase was the aging phenomenon. This type of response has been observed previously for the particulate $NADH_2$ oxidase of *Azotobacter* (14). This increase in specific activity may result from a progressive breakdown or "solubilization" of the membrane structure. Upon prolonged storage at nonfreezing temperatures, it is likely that an "autolytic" action may cause an effect comparable to the "opening" phenomenon observed and described in mitochondria (3). This effect allows for maximal exposure of active sites to substrate and results in a concomitant increase in specific activity of the particulate enzyme preparation.

Distinction from a cytochrome c peroxidase. A further attempt was made to show that the oxidation of mammalian cytochrome *c* by the *Azotobacter* R_3 fraction did not involve a cytochrome *c* peroxidase reaction. The following observations enabled a distinction to be made between the two types of cytochrome *c* oxidation:

(i) The oxidation of cytochrome *c* by the *Azotobacter* R_3 fraction was neither inhibited nor stimulated by the presence of mammalian catalase. Furthermore, the use of ferrocytochrome *c* preincubated with catalase had no effect on the assay In contrast, the cytochrome *c* peroxidase is inhibited by the presence of catalase in the assay mixture (1).

(ii) The *Azotobacter* R_3 fraction possesses a highly active catalase which is capable of decomposing peroxide at a rate in excess of 26.8 μatoms of O evolved per min per mg of protein (8). This would preclude H_2O_2 from being an active reactant in the presence of this enzyme fraction.

(iii) Yonetani (16, 18), who has studied extensively the crystalline cytochrome *c* peroxidase, in bakers' yeast demonstrated that no oxidation of ferrocytochrome *c* could occur unless exogenous peroxide was added. No peroxide was required for oxidation for ferrocytochrome *c* by the *Azotobacter* R_3 electron transport fraction. In addition, according to Smith's method (11), when ferrocytochrome *c* was prepared in the manner used in this study, no peroxide was formed. Therefore, no known source of H_2O_2 can be accounted for that would be involved in the cytochrome *c* reaction.

(iv) The reaction kinetics of cytochrome *c* peroxidation does not follow first-order kinetics (18). In contrast, cytochrome *c* oxidation by the *Azotobacter* R_3 fraction displayed classical first-order reaction kinetics (Fig. 3).

(v) Finally, Yonetani (18) has also reported that ferricytochrome *c* is a *mixed* competitive inhibitor for the cytochrome *c* peroxidase. Although ferricytochrome *c* inhibited *Azotobacter* R_3 cytochrome *c* oxidation (Fig. 5), the inhibition was a *strict* competitive one.

Therefore, it is unlikely that a cytochrome *c* peroxidase is present or involved in cytochrome *c* oxidation by the *Azotobacter* R_3 electron transport fraction. Further, it is concluded that oxidation of mammalian cytochrome *c* by the *Azotobacter* R_3 fraction displayed kinetics similar to that observed for cytochrome *c* oxidation by mitochondria. Moreover, cytochrome *c* oxidation by the *Azotobacter* system does reflect terminal

oxidase function, although it is unknown which type cytochrome components (a_1, a_2, or o) are involved.

ACKNOWLEDGMENTS

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