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ELECTROPHORETIC STUDIES ON CYTOCHROME OXIDASE

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SUMMARY

A modified preparation of cytochrome oxidase has been described. The preparation is extremely low in contaminating hemoproteins. Because it required only a low salt concentration for solubility, it was suitable for electrophoretic studies. Paper electrophoresis resulted in the separation of two fluorescent and three hemoprotein areas. Employing a spray reagent which contained a leuco dye and cytochrome *c*, it was possible to locate active cytochrome oxidase in only one area of the paper. Column electrophoresis of the preparation showed that at least partial separation of the hemoprotein having cytochrome oxidase activity from inactive hemoprotein had occurred. Both hemoproteins fractions exhibited the spectral properties of cytochromes *a* and a_3, and the ratio of these cytochromes was uniform throughout.

INTRODUCTION

The cytochrome "*a*" components of the respiratory chain are defined by two distinct properties, spectral character and enzymic activity. Employing spectral examination, most workers have concluded that cytochromes *a* and a_3 are distinct entities[1-3]. Spectral studies show that cytochrome a_3 is autoxidizable and that it reacts with carbon monoxide and cyanide, while cytochrome *a* is not autoxidizable and is unreactive toward these respiratory inhibitors. In their kinetic studies SMITH[4], CHANCE[5], and LUNDEGARDH[6] noted that in the steady state the absorption spectra of the two cytochromes could be altered to different extents. It was also observed[3,7,8] that the molar ratio of cytochrome a_3 to cytochrome *a* varied according to the tissue studied, this ratio being 2.3 in *Bacillus subtilis* and 1.0 in yeast and heart muscle. Further evidence in support of the separate existence of cytochrome a_3 is noted in

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the fact that the carbon monoxide-reduced difference spectrum of cytochrome oxidase agrees well with the action spectra determined by CHANCE[9] and by WARBURG AND NEGELEIN[10] for the "respiratory ferment".

Although many workers have made partial separations of cytochromes *a* and a_3 from the other pigments of the respiratory system[11–16], attempts to separate cytochrome *a* from cytochrome a_3 have been unsuccessful. WAINIO and coworkers[17–19], employing serial fractionation with bile salts, were able to separate cytochromes *a* and a_3 from cytochrome *b* but not from each other. The use of proteolytic enzymes followed by salt fractionation resulted in a purified oxidase which, however, still displayed the properties of both cytochromes *a* and a_3[14]. Several workers[20–22] subjected their cytochrome preparations to centrifugal forces up to 260,000 *g*, but no evidence could be found for the separation of cytochrome *a* from a_3. This lack of success has been interpreted as evidence for a single "*a*" type cytochrome[18].

One of the most useful techniques for the study of protein homogeneity is zone electrophoresis. This paper describes experiments in which a soluble cytochrome oxidase preparation is subjected to paper and column electrophoresis in an effort to investigate the protein composition of the preparation. The components resulting from these electrophoretic separations were analyzed by spectral and enzymic methods.

MATERIALS AND METHODS

Enzyme preparation

The cytochrome oxidase preparation of SMITH AND STOTZ[14] is not suitable as a beginning material for electrophoretic work. With certain modifications, however, this procedure will yield a protein solution which remains soluble under the conditions suitable for electrophoresis.

The modified preparative procedure is as follows. The macroscopic fat and connective tissue were removed from pig heart muscle. The meat was ground twice and 200 g were washed five times with 2 l cold distilled water. The tissue was squeezed in shroud cloth after each washing to remove excess water. The mince was then homogenized with 500 ml cold 0.1 *M* phosphate buffer, pH 7.4, for 7 min in a Waring blendor. Centrifugation of the slurry in an International refrigerated centrifuge for 45 min at about 2000 *g* separated the heavier particles from a cloudy supernatant fluid which was decanted and saved. The sediment was then reextracted with 300 ml buffer by homogenizing for 2 min in the Waring blendor. After centrifuging this suspension at 2000 *g* for 45 min, the supernatant fluid obtained was combined with the first extract. The total volume was usually about 630 ml.

The pH of the heart muscle extract was lowered to pH 5.6 by the addition of about 20 ml cold 2 *M* acetic acid. The precipitate which formed was immediately collected by centrifugation at 2500 *g* for 1 h in the cold. The clear red supernatant fluid was removed by aspiration and the residue was resuspended in about 200 ml cold distilled water. The suspension was then centrifuged for 15 min at 5000 *g* and the residue was suspended in the pH 7.4 buffer to a final volume of 300 ml. A hand operated ground glass homogenizer was used to disperse any clumps.

2.0 ml of a 40 % neutral sodium cholate solution and 25 mg crude trypsin were added for each 100 ml suspension. These reagents were the same as those previously described[14]. The mixture was allowed to stand at 4° for 1 h with frequent stirring.

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26 g solid ammonium sulfate were then added slowly to each 100 ml of the solution (Soln. A) which resulted in a 0.45 saturation. The mixture was allowed to stand in the cold for 3 h. The precipitated protein was then separated by centrifugation in the cold at 7000 *g* for 20 min in a Servall high speed centrifuge. The ammonium sulfate concentration of the clear supernatant fluid was brought to 0.54 saturation by the addition of 6 g solid salt per 100 ml of the original solution (Soln. A), and after standing for 15 min the precipitate was centrifuged as above. The precipitate was dissolved in 30 ml 0.1 *M* disodium phosphate.

Soluble cytochrome oxidase prepared in this manner was first subjected to experiments designed to lower the ionic strength to the minimum required for solubility. The preparation contains approximately 3 % ammonium sulfate in addition to smaller amounts of sodium cholate and disodium phosphate. This amount of salt provides an ionic strength which is unsatisfactory for electrophoretic separation of proteins. Efforts to lower the ionic strength were directed toward the removal of ammonium sulfate while maintaining conditions necessary for enzyme solubility by increasing the concentration of sodium cholate. By dialyzing portions of the preparation against phosphate buffer containing various concentrations of cholate, it was found that after complete removal of the ammonium sulfate the active enzyme remained soluble over extended periods in the cold in 0.1 *M* phosphate buffer, pH 7.4, containing 2 % sodium cholate. Consequently, for electrophoresis, in the final step of the preparative procedure the 0.45–0.54 precipitate was dissolved in this solution and dialyzed in the cold against 20 volumes of the same solution for 6 h with three changes of solvent.

Electrophoresis

Paper electrophoresis employing Whatman 3 mm paper was conducted according to the technique previously described[23]. The electrode chambers were filled with a solution consisting of 2 % sodium cholate in 0.1 *M* phosphate buffer, pH 7.4. From 0.05 ml to 0.10 ml protein solution was applied to the paper by streaking. Electrophoresis was carried out at 4° for about 12 h using 225–300 V which provided a flow of current of 11.5–14 mA.

The distribution of proteins on the paper strip was determined by the use of bromophenol blue reagent[23]. The hemoproteins were located with a benzidine–peroxide spray reagent[24].

In order to locate the protein having cytochrome oxidase activity the paper was sprayed immediately after electrophoresis with a reagent designed for rapid qualitative detection of the enzyme. The reagent contains a leuco dye which in the presence of cytochrome *c* is rapidly oxidized to a blue compound by cytochrome oxidase. This reaction is not catalyzed by heat-denature dcytochrome oxidase, cytochrome *b* or cytochrome c_1. The rate of autoxidation of the dye is sufficiently slow that it does not interfere with the location of the active enzymes. The reagent is prepared as follows: 5.0 ml water, 10 ml 0.1 *M* phosphate buffer, pH 7.4, and 1.5 ml $2 \cdot 10^{-4}$ *M* cytochrome *c* were mixed in a 50 ml sprayer flask and the oxygen was flushed out with nitrogen or hydrogen gas. Just prior to use, 3.0 ml 0.35 % 2,6-dichlorophenone-indo-3'-chlorophenol (Eastman Kodak), which had been reduced to the colorless state by hydrogen gas with a palladium–asbestos catalyst, were added to the flask. Hydrogen was bubbled through the solution for a few minutes to fill the flask with this gas and the flask was stoppered. The area which contains active cytochrome

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oxidase appears blue within a few seconds after being treated with the reagent. If nitrogen is used to supply the pressure for spraying, the dye remains suitable for use for several hours.

Column electrophoresis was conducted using an LKB apparatus according to the procedure described by SVENSSON[25]. The supporting medium consisted of methylated cotton cellulose[26], used in order to minimize adsorption. 5.0 ml protein solution were admitted to the column, and following electrophoresis at 250 V and 95 mA, the separated proteins were washed from the column and collected with an automatic fraction collector. During the course of these procedures the movement of both fluorescing and absorbing bands was followed by the use of ultraviolet light.

Spectra

Visual spectra of the oxidized, reduced, and carbon monoxide treated cytochrome oxidase were measured by a recording spectrophotometer (Warren Spectrocord). Reduction of the enzyme was accomplished by the addition of a few mg solid sodium dithionite. The carbon monoxide complex was prepared by passing carbon monoxide gas through the reduced enzyme solution for 2–3 min.

Enzyme assay

The determination of cytochrome oxidase activity was carried out as previously described[27]. Enzymic activity is expressed as $\Delta \log I$/min/mg protein. The quantities of protein present in various samples were determined either by a turbidimetric method[28], which measures optical opacity after addition of sulfosalicylic acid, or by measuring the extinction of the protein solutions at 280 mμ.

Paper chromatography

Chromatographic investigation of flavin components was conducted according to the method of KILGOUR *et al.*[29], using one solvent system composed of *n*-butanol–acetic acid–water (40:10:50) and a second system composed of *tert.*-butanol–pyridine–water (60:15:25). Flavin spots on the developed papers were located under ultraviolet light.

RESULTS

The preparation of cytochrome oxidase obtained as described above is a clear olive-green solution. Fig. 1 shows the visible spectra of the oxidized, reduced, and carbon monoxide treated enzyme. In addition to the 605 mμ and 444 mμ peaks of the reduced preparation there are minor absorptions at 520 mμ and 560 mμ. These latter peaks are apparently due to hemoprotein contaminants of cytochrome oxidase[14].

The addition of carbon monoxide affects both the 605 mμ (α) and 444 mμ (γ) absorption peaks, and in addition a β-peak appears in the region of 550 mμ. This latter peak is more obvious in the difference spectrum* shown in Fig. 2. In this spectrum, the curve above the axis, which represents the cytochrome a_3-carbon monoxide complex, has maxima at 428, 545, and 590 mμ, with the suggestion of

* The carbon monoxide difference spectrum will refer to the absorbancy of the reduced compound treated with carbon monoxide minus the corresponding absorbancy of the reduced compound.

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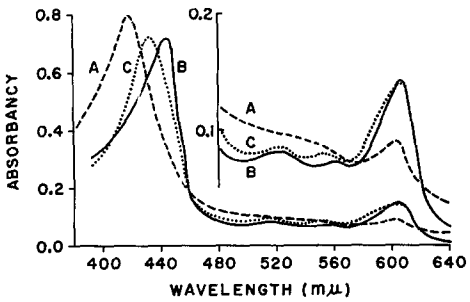

Fig. 1. The visible spectra of cytochrome oxidase. Curve A represents the spectrum of the oxidized preparation in 0.1 *M* phosphate buffer, pH 7.4, containing 2% cholate. Curve B represents the same preparation reduced with 2 mg sodium dithionite. Curve C represents the preparation reduced with dithionite and treated with carbon monoxide.

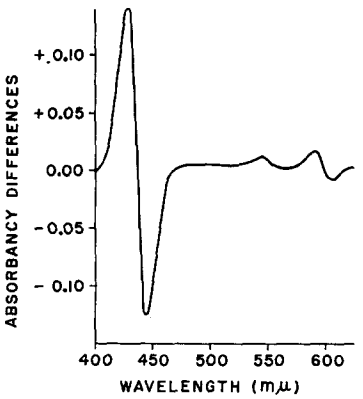

Fig. 2. The carbon monoxide difference spectrum of cytochrome oxidase.

a shoulder on the low wavelength side of the 545 mμ peak. The curve below the axis has maxima at 444 and 605 mμ and represents reduced cytochrome a_3.

Typical results of paper electrophoresis experiments are shown in Fig. 3. Areas H1, H2, and H3 give positive reactions with the benzidine spray and are stained by bromphenol blue. Consequently these areas are considered to contain hemoproteins. Occasionally non-pigmented proteins were found just short of area H1 and well in advance of H3. Of the three hemoproteins, H2 was the most concentrated, H1 was next most concentrated, and H3 was present in lowest concentration. Sometimes a hemoprotein band appeared very near the origin, but this finding was inconsistent.

Cytochrome oxidase activity was observed in only one area of the paper. It appeared on the leading portion of hemoprotein area H2, as shown by the hatched marks in Fig. 3.

Two fluorescing bands F1 and F2 appeared under ultraviolet light. The stronger of these, F2, was located just ahead of the slowest moving hemoprotein (H1) and the second, F1, was observed to have moved only slightly away from the origin. Paper chromatography of F2 showed it to move as a riboflavin compound.

In order to obtain sufficient material for quantitative measurements, the enzyme preparation was subjected to column electrophoresis. After separation the samples were eluted and the fractions collected were investigated for their protein and hemoprotein concentrations, cytochrome oxidase activity, and fluorescence. Fig. 4 shows typical results of such studies. A small protein peak always appeared just ahead of the main band. The hemoproteins as measured by the absorption at 410 mμ, were contained within a single peak. The fluorescing compounds, on the other hand, were separated by the column procedure in a manner similar to that obtained using paper.

A comparison of curves A and A′, which represent protein concentration, with curve B, which represents hemoprotein concentration, shows that the hemoprotein is contained in the forward portion of the major protein peak. A plot of the enzymic activity (curve C) locates the active enzyme also in the forward portion of the protein peak. The enzymic activity (curve C) is associated with the material having the

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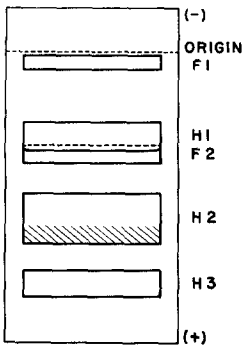

Fig. 3. The paper electrophoretic separation of hemoproteins in the cytochrome oxidase preparation.

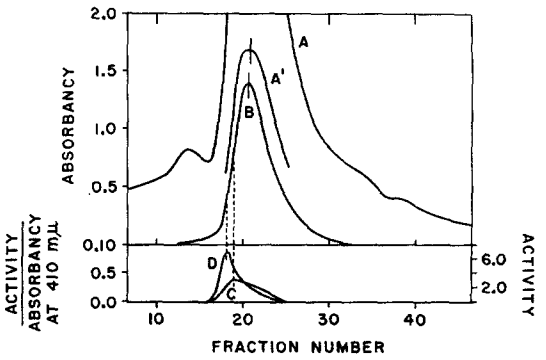

Fig. 4. Analysis of the fractions obtained from the column electrophoresis of the cytochrome oxidase preparation. Each fraction represents 3 ml. Curve A shows the absorbancy of each fraction at 280 mμ and curve A′ the absorbancy at 280 mμ of fractions 18–25, diluted three-fold. Curve B represents the absorbancy of the undiluted fractions at 410 mμ. Curve C shows the distribution of enzymic activity among the different fractions. Curve D is the ratio of enzyme activity units to absorbancy at 410 mμ.

greatest electrophoretic mobility of the hemoproteins present (curve B), hence it appears that much of the hemoprotein present is not active cytochrome oxidase. This is clearly illustrated by curve D which represents the ratio of oxidase activity to absorbancy at 410 mμ.

Spectra of the oxidized, reduced and carbon monoxide treated forms of the fractions having activity were recorded. An analysis of the spectral data showed that the positions of the absorption maxima of oxidized, reduced, and carbon monoxide compounds were the same as those of the preparation before electrophoresis. Furthermore, the maxima of the carbon monoxide difference spectra were also identical with those observed prior to electrophoresis. Despite the fact that there appeared to be a fractionation of enzymatic activity with respect to hemoprotein concentration, no parallel changes in spectral properties were observed.

Since the enzyme system under investigation is usually considered to require at least two components, cytochromes a and a_3, it was considered possible that the distribution of enzymatic activity relative to the hemoprotein concentration might reflect a partial separation of these components during the course of electrophoresis. Table I shows the activity of some individual fractions and the activity resulting from the combination of some of these fractions. It can be noted in all instances that the activity of the combined fractions is strictly additive.

It should also be pointed out that nearly 2/3 of the total oxidase activity was lost during electrophoresis. A similar loss of activity was observed in samples of the preparation which were kept under identical conditions, but not subjected to the electrophoretic procedure. It can therefore be concluded that the electrophoresis was not responsible for this inactivation.

DISCUSSION

The investigation of the composition of previously described preparations of cytochrome oxidase has been seriously hampered by the presence of a contaminating

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TABLE I

THE CYTOCHROME OXIDASE ACTIVITY OF FRACTIONS OBTAINED BY ELECTROPHORESIS, ALONE AND IN COMBINATIONS

The fractions were those obtained from an electrophoretic separation similar to that shown in Fig. 4. Units of enzyme are as defined in the text.

Fraction number	*Enzyme activity (units)*
15	1.2
16	5.4
17	3.1
19	0.6
20	0.2
21	0.2
15 + 20	1.4
15 + 21	1.4
16 + 19	6.0
17 + 20	3.3

hemoprotein and a requirement of high salt concentration for solubility. Some success in removing the contaminating hemoprotein has been achieved employing either precipitation[14] or adsorption[4] techniques. By employing a modified ammonium sulfate fractionation, which introduces no additional steps, we have eliminated the major portion of the contaminating hemoprotein. Purity of the preparation as judged by the protein to heme ratio (280/444 mμ) appears to be as high or higher than that achieved by others using involved purification procedures. This preparation also has an added advantage in that it does not require high ionic strength for solubility as is the case of other preparations. These conditions made possible the application of electrophoresis to this soluble cytochrome oxidase.

Paper electrophoresis resulted in the separation of several proteins. Since it has been established that different oxidation states of a hemoprotein can be separated on the basis of charge[30], this possibility must be considered. However, since the enzymic activity was contained in a single area, it seems unlikely that the components were separated solely on the basis of their states of oxidation.

The partial separation of active from an inactive cytochrome oxidase appears to have been accomplished using column electrophoresis. However, a systematic study of the carbon monoxide difference spectra failed to yield any evidence for the separation of cytochromes *a* and a_3. All fractions containing activity exhibited the spectra attributed to both pigments, and the apparent proportion of cytochrome *a* to cytochrome a_3 remained constant even though the enzymic activity per unit of protein varied markedly. The additive nature of the enzymic fractions indicates that the rate limiting step in the oxidase reaction was the same in all hemoprotein fractions. The observations taken together would imply that the green inactive protein having a lower electrophoretic mobility than the active protein, is probably denatured oxidase.

The electrophoretic movement of active cytochrome oxidase in pH 7.4 buffer indicates that its isoelectric point is somewhat below this pH, probably in the region of pH 6.0. It is noted that the oxidase is precipitated during the preparation procedure at pH 5.6.

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The attempts to separate the components of cytochrome oxidase by means of electrophoretic methods, although not entirely successful, indicate the potential of the method. The hemoproteins of cytochrome oxidase have been partially separated from the non-pigmented protein and the active enzyme has been partially separated from the inactive protein. Thus it appears that with further modifications, perhaps in regard to the physical state of the preparation or in regard to the medium, more resolution might be obtained.

ACKNOWLEDGEMENTS

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