ON THE PHYSICAL-CHEMICAL PROPERTIES OF WATER-SOLUBLE CYTOCHROME OXIDASE

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The isolation by D. E. Green's group at the Institute for Enzyme Research of some of the component enzymes of the mitochondrial electron transport chain enabled us to study the possibility of obtaining these as single, active, molecularly dispersed entities. In this connection we have studied a variety of protein and lipoprotein components and have developed procedures which lead to molecularly dispersed cytochrome $\underline{c}_1$ and cytochrome oxidase.

Cytochrome $\underline{c}_1$ as prepared by Green *et al.* (1959) appeared to be a single, lipid-free, homogeneous compound with a molecular weight of 371,000. Cytochrome $\underline{c}_1$ has also been prepared in a form containing 10 to 15% bound lipid and with a molecular weight of 410,000 (Gilboe, 1958). Our recent studies indicate that when lipid-free $\underline{c}_1$ is treated with sodium thioglycolate at basic pH's and in the presence of detergent an enzymatically active species containing a single heme group per molecule is formed. The molecular weight, determined by Ehrenberg's modification of the Archibald procedure (Ehrenberg, 1957), is approximately 70,000. Thus the parent cytochrome unit probably contains at least 5 molecules of cytochrome $\underline{c}_1$ each of molecular weight 70,000.

The present communication deals with the effect of a similar thioglycolate treatment on the physical properties of particulate cytochrome oxidase. The details of the preparation of water-

soluble cytochrome oxidase from the usual particulate preparation are given in an accompanying paper (Ambe and Venkataraman, 1959).

Centrifugation of the soluble preparation in the Model E Spinco ultracentrifuge at 59,780 rpm in pH 8.5 tris(hydroxymethyl) aminomethane acetate buffer (.001 M tris) plus NaCl to bring ionic strength to 0.05 showed five to six distinct sedimenting components. That the characteristic cytochrome oxidase spectra moved with all of the peaks was shown by experiments using a partition cell. This indicated the possibility of a polymerizing or aggregating system. Confirmation of this hypothesis came from a variety of sources. When the protein solution was allowed to stand for a week or two a slow increase in the concentration of the faster sedimenting components with a corresponding decrease in concentration of slower sedimenting components was observed. Moving boundary electrophoresis also gave added support since the soluble cytochrome oxidase was observed to migrate the entire length of the cell with a single, symmetrical schlieren peak, indicating a single charge to mass ratio in the preparation.

Further treatment of cytochrome oxidase with thioglycolate in the presence of deoxycholate, 6 M urea, or triton X-100 using the same reaction conditions as outlined in the accompanying paper was shown to have little or no effect in further reducing the size of the aggregate. Treatment with thioglycolate followed by addition of iodoacetate to prevent reformation of any ruptured disulfide bonds also proved ineffective in producing a monomeric form. The readdition of deoxycholate or triton X-100 seemed to have little effect on the soluble preparation, but sodium dodecyl sulfate (SDS) in quantities as low as .001 M caused an immediate conversion of the aggregate to the monomeric form plus some dimer. As the concentration of SDS was increased from 0.001 M to 0.1 M the concentration of monomer increased with a corresponding decrease

in the dimer concentration. At 0.001 M SDS there was approximately 70% monomer and 30% dimer, while at 0.1 M SDS the ratio was 95% monomer and 5% dimer.

In order to avoid uncertainties in the effects of high detergent concentrations upon protein molecules, molecular weight determinations were carried out in 0.002 M SDS where the protein was still 15% in the form of the dimer. To determine molecular weight, Ehrenberg's (1957) modification of the Archibald method of approach to sedimentation equilibrium was used. All runs were made at 17,980 rpm at pH 8.5 in 0.001 M tris acetate buffer. The concentrations of the protein at the meniscus and the total concentration were determined by planimetering a photographic enlargement of the area under the schlieren pattern. To determine total concentration, a synthetic boundary forming cell was employed. The results of this experiment, which are shown in Fig. 1, indicate

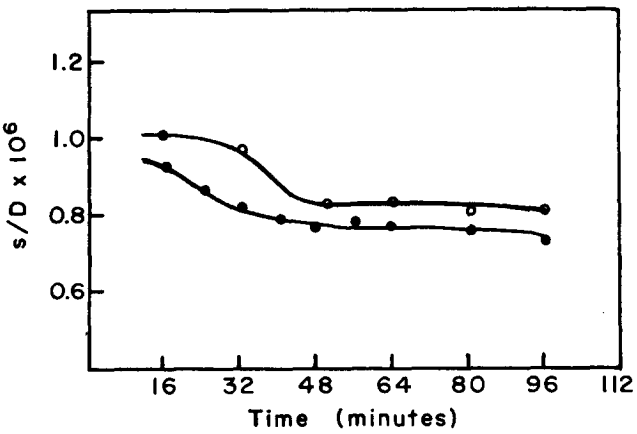

Figure 1

Molecular Weight Determination of Cytochrome Oxidase (-●-) and Mercuric Albumin Monomer (-○-).

that the ratio s/D (which is proportional to molecular weight) initially decreases with time but then levels off for a period of approximately 40 minutes. The shape of this curve corresponds to that predicted for a system which equilibrates slowly compared to the duration of the experiment and closely resembles the results observed for a known artificial mixture of 90% serum albumin plus

10% mercuric albumin dimer. It is expected that initially the weight average molecular weight at the meniscus would decrease with time due to the pulling away from the meniscus of the dimer. The leveling of the curve is an indication that the molecular weight at the meniscus is no longer changing appreciably and that we are approaching a condition where only the monomer is present and being measured. The best average value for the ratio s/D corresponding to the plateau region is 7.7×10^{-7}.

The apparent partial specific volume ($\bar{v}$) of cytochrome oxidase was measured using the falling drop method of Linderstrøm-Lang and Lanz (1938). A linear density gradient was formed using water saturated solutions of n-chloro butane and chlorobenzene mixed according to the method of Bock and Ling (1954) and thermostated at 20 $\pm$ 0.01 degrees during all measurements. Reference density solutions were made up using potassium chloride. The protein concentration was determined by measuring the dry weight of a sample which had been lyophilized for 24 hours and then dried at 60° for 24 hours in vacuo. This gave a $\bar{v}$ of 0.74 cm^3 $gram^{-1}$ for cytochrome oxidase.

Combining these terms in the familiar Svedberg equation, $M = \frac{RTS}{(1-\rho\bar{v})D}$, we arrive at a molecular weight of 72,000.

Since the spectral properties of cytochromes are so distinctly characteristic the effect of SDS on spectra was also observed. It was noted that the only change induced by the detergent was a shift of the normal alpha peak from the usual 603 mμ to 595 mμ with a slight broadening of the peak.

The correspondence of the molecular weight determined from centrifugal data with that obtained as the minimal molecular weight from heme, iron and copper content is discussed in the accompanying paper.

In summary, while a combination of disulfide-breaking and

surface-active reagents rendered cytochrome oxidase water soluble, a further treatment with sodium dodecyl sulfate was necessary to disperse the active enzyme into a state bearing only one heme, one iron and one copper per molecular unit in solution. The principle that sub-mitochondrial enzymes can be rendered water-soluble, active and molecularly dispersed by an appropriate combination of agents known to break selected disulfide, hydrophobic and electrostatic bonds now appears well established.

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