ON THE STABILITY OF THE LATTICE STRUCTURE OF MEMBRANOUS CYTOCHROME OXIDASE

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Summary: The two dimensional crystal lattice formed by the protein complexes in membranous cytochrome oxidase has been observed under a variety of staining conditions, including in the absence of any stain or fixative. The crystal lattice was reversibly destroyed at pH values above 8.3, in high salt and under reducing conditions. These results indicate that the lattice structure of the cytochrome oxidase membrane is very sensitive to the conformation of the enzymic complex, and that protein-protein interactions are involved in stabilizing the lattice structure.

Introduction

We have previously shown[1] that membranous cytochrome oxidase prepared by the method of Sun et al.[2] displays a regular surface two-dimensional lattice structure when examined electron microscopically. Using the lattice constants measured from the micrographs, together with the known chemical composition of the material, it was possible to construct a geometrical model for the membrane which gave quantitative agreement with the available data. In this model, the cytochrome oxidase protein complexes form a two-dimensional crystalline array, with the phospholipids filling the spaces between the proteins as lipid bilayer. The present paper will give the results of additional studies to determine the factors which contribute to the stability of the lattice structure. These include the effect of pH, various salts, redox state, and temperature.

Methods

The preparation of cytochrome oxidase and the electron microscopic methods were as previously reported (1).* In most experiments, fixation in solution

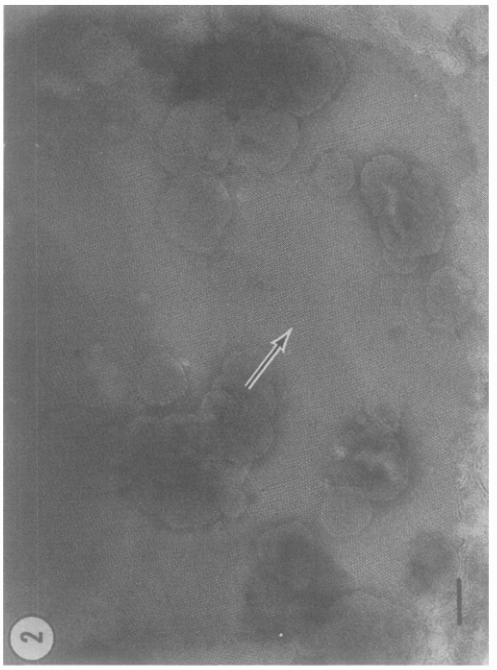
*The pH of the preparation was erroneously reported in the previous paper (1) to be 7.8 instead of 7.4 as intended. The preparation works well as either pH 7.0 or 7.4, but not at pH 7.8 or above.

with 0.05% or 1.0% (final concentration) glutaraldehyde was followed by staining with 1% uranyl acetate (pH 4.0) on a carbon-coated copper grid. Up to 2% glutaraldehyde was used in experiments to test the effect of this reagent, while in other experiments either the glutaraldehyde treatment or the staining or both were entirely omitted. The effects of pH and different salts were studied by suspending a portion of the oxidase in media of a series of hydrogen ion or salt concentrations, at 0-4°C. For the pH studies, Tris-HCl buffers (50 mM) augmented with 0.25 M sucrose were used. In the salt studies, the pH was maintained at 7.2 with Tris-HCl (10 mM), to which was added various concentrations of the specified salt (NaCl, NaBr, $CaCl_2$ and $MgCl_2$). In a separate experiment, 5 mM EDTA was added in the presence of 10 mM Tris-HCl (pH 7.2) plus 0.25 M sucrose, to remove divalent ions. Reversibility of the effect of 1.2 M NaCl was studied by dialyzing overnight against distilled water to remove the excess salt. The effect of reducing conditions on the lattice was studied by reduction at pH 7.0 in the presence of 1.0 mM KCN with powdered dithionite in a nitrogen atmosphere, or by adding reduced cytochrome c. Reversibility of the cytochrome c reduction was studied by permitting reoxidation by oxygen. The effect of temperature was studied by incubating a sample of the cytochrome oxidase for 5 min at the desired temperature, followed by fixation at that temperature with 1.0% glutaraldehyde; the solution contained 10 mM Tris-HCl plus 0.25 M sucrose, at pH 7.2.

Results and Discussion

Effect of Different Staining Conditions. The lattice structure could be observed under all staining conditions tested; these included no staining whatsoever, phosphotungstic acid stain, and uranyl acetate stain. The same results were obtained whether or not the specimen was pre-fixed with glutaraldehyde. Uranyl acetate staining gave the clearest pictures and was routinely employed for most of the work reported here.

Figure 1 shows the appearance of unstained membranous cytochrome oxidase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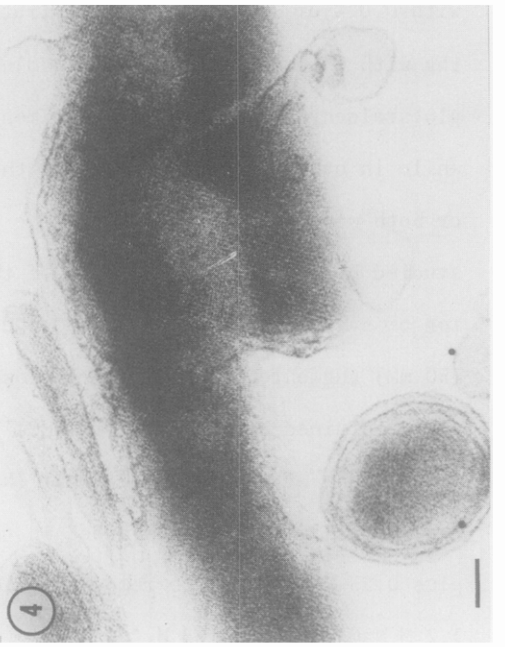
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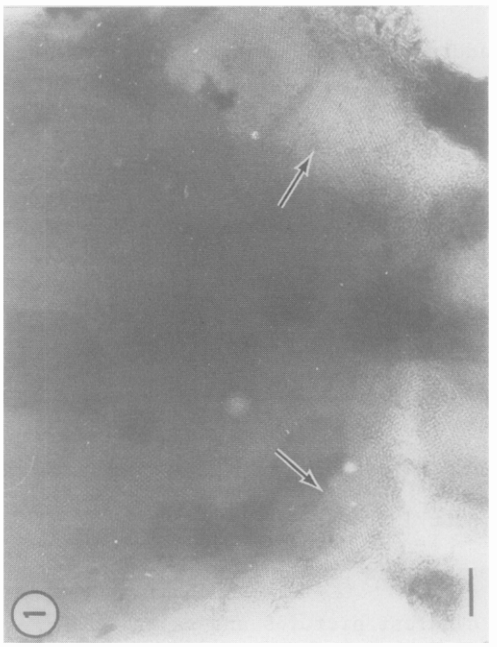
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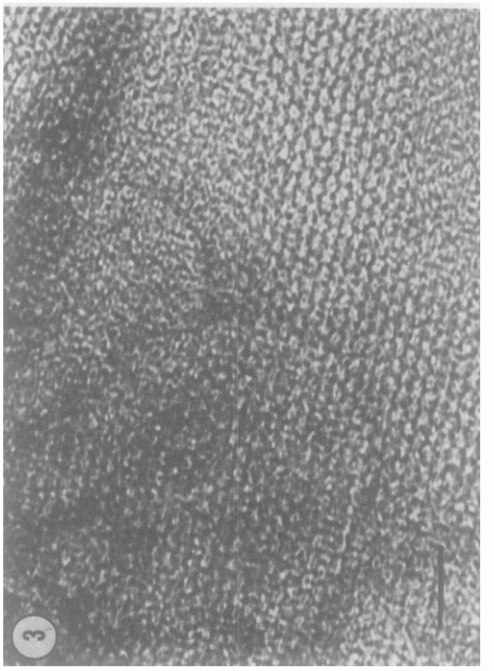
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deposited on a carbon-coated copper grid, and for comparison Fig. 2 shows a preparation stained with uranyl acetate in the usual manner. No glutaraldehyde was used in either case. As one might expect, the contrast in Fig. 1 is inferior to that of Fig. 2, but the lattice structure is definitely present in both cases. This conclusively demonstrates that the observed lattice structure is not induced by staining. Fig. 3 shows the highest resolution micrograph yet obtained, while in Fig. 4 is shown a thin section of OsO_4-stained material, made at an oblique angle to the stacked aggregate of membranes. In the oblique section, a lattice pattern which corresponds to the surface lattice seen by negative staining is visible.

Hydrogen ion concentration. The effect of pH was monitored by taking up aliquots of cytochrome oxidase in buffered media from pH 6.4 to 9.0 in approximately 0.5 pH-unit steps. (Since Tris-HCl was used, the solution was essentially unbuffered at the lowest pH value.) The lattice structure remained stable from pH 6.4 to 8.0, but became unstable (as inferred from the electron microscopic appearance) in the range of pH 8.0 to 8.5, with no lattice structure being seen at or above pH 8.5. The integrity of the membrane was retained throughout the pH range studied. Reversibility of the pH effect was demonstrated by adding HCl to a pH 8.5 solution so as to lower it to pH 7.0; the lattice structure was regained after standing for about an hour at

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Figure 1. Cytochrome oxidase membrane dried on a carbon coated copper grid without addition of either fixative or stain. The contrast is poor, but the lattice structure can nonetheless still be seen. Distance marker = 1000 Å, scope magnification = 25 K.

Figure 2. Electron micrograph of cytochrome oxidase membrane surface with uranyl acetate, showing the long range order of the lattice structure. The distance marker equals 1000 Å; the scope magnification was 25 K.

Figure 3. High resolution micrograph of membranous oxidase stained with uranyl acetate. The irregularly shaped white spots are the protein complexes, which measure approximately 50 x 60 Å. The scope magnification in this case was 312 K; the distance marker equals 500 Å.

Figure 4. Thin-sectioned cytochrome oxidase membranes, which were fixed in 1% glutaraldehyde, exposed to OsO_4 for 1 hr, and stained with 1% uranyl acetate (1). The section made an oblique angle with the plane of the membrane. Note presence of lattice structure. Distance marker = 1000 Å; scope magnification = 25 K.

the lower pH value. The fact that the lattice structure is lost in the same pH range as the enzymic activity is lost (3), suggests that the same pH-dependent conformational changes which presumably cause loss of activity may also be responsible for the loss of crystallinity. The crystallinity, however, is definitely not a requirement for activity, as shown by other experiments.

Salt effects. It was found that moderate levels of neutral salts (NaCl and NaBr) had no effect on the lattice, but that concentrations at or above 1.2 M NaCl or 0.7 M NaBr caused disappearance of the lattice structure. No protein was extracted by the high salt treatments. Dialysis against distilled water following incubation in 1.5 M NaCl slowly gave restoration of the lattice structure. Whereas high salt was detrimental, removal of salt by dialysis against distilled water, or removal of divalent metal ions through the addition of 5 mM EDTA, did not destroy the lattice. Also, the addition of 5 mM Ca^{++} or Mg^{++} did not affect the lattice. The high levels of neutral salts required to affect the lattice are in the ranges known to affect membranes and proteins in a generalized chaotropic manner (4); this is also indicated by the lower concentration of bromide than chloride required to produce the same effect, since bromide is known to be a stronger "chaotrope" than chloride (4).

Oxidation-reduction. It has previously been reported by Wakabayashi et al. (5) that the lattice structure is abolished by reduction of the enzyme. We have confirmed this observation, and have also shown that the lattice structure is regained upon oxidation by aeration following the reduction with cytochrome c. It is known that protein conformational changes accompany the oxidation-reduction reaction (6). These same conformational changes must be responsible for the loss of crystallinity.

General Conclusions. Our results with a number of different perturbants of protein structure indicate the sensitivity of the lattice structure to the

conformation of the enzymic complex. It appears that the native, oxidized conformation of cytochrome oxidase is required for crystallinity.

The effects of the various perturbants also suggest that protein-protein interactions between complexes are important in stabilizing the lattice structure. These interactions are apparent in the high magnification micrograph (Fig. 3) and can be visualized clearly in optically filtered images of our micrographs, which show each complex linked to its six nearest neighbours (7).

Finally, our data suggests that the lattice structure of the cytochrome oxidase membrane may be a property of the membrane in aqueous milieu. Glutaraldehyde fixation has been shown to freeze effectively the organization of membranes in the solution state (8,9). Glutaraldehyde treatment of our membrane preparation (suspended in 10 mM Tris HCl pH 7.2) preserves the lattice structure through the drying steps required for electron microscopy, although the reagent itself does not induce the regular array. X-ray diffraction studies of cytochrome oxidase membranes are currently in progress to determine conclusively whether an aqueous suspension of the membrane shows the lattice structure we have observed by electron microscopy.

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