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Effect of Reduction and Solubilization on the Conformation of Cytochrome Oxidase

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Abstract

Membraneous cytochrome oxidase (E.C. 1.9.3.1) was labelled with iodoacetamide and maleimide spin labels and solubilized with Triton X-100. On reduction, the EPR spectrum of the original membraneous oxidase was shifted toward the less strongly immobilized form as measured with either label.

After solubilization, the EPR spectrum in the oxidized state was unchanged. The change of conformation upon reduction was either eliminated (if measured with iodoacetamide label) or was reversed, i.e., changed toward the strongly immobilized spectrum, if measured with maleimide label.

This finding indicates that solubilization does not alter the conformation of the oxidized form of cytochrome oxidase, but does change the preferred conformation of the reduced form.

Introduction

Recent studies on the conformation of cytochrome oxidase using optical rotatory dispersion and circular dichroism[1–5] have shown that cytochrome oxidase has distinct conformations in the oxidized and reduced states, which are modified in the presence of inhibitors. In particular, Myer and King[1] showed that when oxidized soluble cytochrome oxidase was aged or treated with desoxycholate, there was little effect on the conformation of the oxidized enzyme but a strong effect on the conformation of the reduced enzyme.

We have studied the conformation of cytochrome oxidase with the spin label technique,[6,7] using two preparations of cytochrome oxidase developed in this laboratory by Jacobs and co-workers.[8–10] The membraneous preparation is probably more "native" than the preparations used by Myer and King, while the solubilized preparation is probably very similar to their Emasol preparation.

The present paper concerns itself primarily with the effects of reduction and solubilization. Effects of inhibitors will be reported later.

Materials and Methods

Rat liver mitochondria were prepared essentially according to Lardy and Wellman.[11] Three types of cytochrome oxidase preparation were made from these mitochondria.

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High-lipid membraneous cytochrome oxidase was made as described in Jacobs *et al.*[8] by dissolving mitochondria in a solution of 1·3% Triton X-114 and 0·2 M neutral potassium phosphate. This preparation could be converted into low-lipid membraneous cytochrome oxidase by repeated washing with 0·05 M phosphate. The low-lipid form can also be prepared directly according to the procedure in ref. 10, using 2% X-114 and 0·05 M phosphate. No differences were found between these two forms with regard to conformation changes, so the low-lipid preparation was generally used as a matter of convenience and will be referred to as membraneous oxidase below.

Soluble cytochrome oxidase can be prepared directly from the high-lipid form[9] or from the low lipid form. In the latter case a solution of 5% Triton X-100 and 0·2 M phosphate was used for solubilization, and a white, heme-free membraneous or aggregated protein was obtained in addition to the soluble cytochrome oxidase. This protein, which constitutes about 40% of the protein before solubilization, is not observed when the high-lipid form of membraneous cytochrome oxidase is solubilized, but appears when a high-lipid preparation is delipidized and freed of Triton X-114 by washing. This protein, which will be called "residue" below, is probably a form of structural protein, although the possibility that it is apo-cytochrome oxidase has not been excluded.

Iodoacetamide spin label, (2,2,6,6-tetramethyl-1-oxyl-4-piperidyn1) iodoacetamide, was prepared by a method of Ogada.[12] Maleimide spin label, *N*-(2,2,6,6-tetramethyl-1-oxyl-4-piperidyn1) maleimide, was a gift of Dr. W. C. Landgraf of Varian Associates, and of Dr. H. McConnell of Stanford. Structural formulae of these labels may be found in refs. 6 or 7.

Membraneous oxidase was labelled by incubating it over night with a large excess of iodoacetamide or maleimide spin label in 0·05 M phosphate, pH 7·5, at 0–4°. Spin label was added at a ratio of approximately 1 mg label per 100 mg or less of protein in order to saturate all available sulfhydryl groups. The preparation was then washed three times in 0·05 M neutral phosphate to remove excess spin label, and taken up as a concentrated suspension for immediate measurements, or as a more dilute one for solubilization. Oxidase was not labelled after solubilization primarily because the two days required to dialyse excess label away frequently resulted in denatured preparations. Bovine serum albumin was labelled by incubation as above, and excess label was removed by dialysis.

Electron paramagnetic resonance (EPR) measurements were taken on a Varian V-4502 spectrometer in standard aqueous cells at room temperature. For anaerobic (reduced) measurements, a vessel similar to that described by Palmer[13] was attached to the aqueous cell.

Optical spectra were measured on a Cary 14 spectrophotometer, either in standard 1 cm cells or in the aqueous EPR cell during experiments. Measurements in the standard cells were of much better quality, and measurement of solutions in the aqueous EPR cells was generally used only to confirm that solutions that looked reduced to the eye were not partially oxidized. The optical spectrum of cytochrome oxidase was not changed by spin labelling.

As a control, activity measurements were made with a Clark oxygen electrode, as described in refs. 8 and 9. The activity of the oxidase was not changed by spin labelling.

Protein was measured with the biuret reagent of Gornall *et al.*[14] and corrected for heme adsorption by the method of Yonetani.[15]

Experiments and Results

Since nitroxide spin labels are vulnerable to reduction of the nitroxide group, which abolishes the free radical that causes the EPR spectrum,[6] careful choice of a reduction system for the cytochrome oxidase was necessary. Ascorbate and dithionite, the most widely used reductants for cytochrome oxidase, reduce spin labels almost instantaneously. Reduction of the labels with NADH was much slower, requiring several hours to reduce 90% or more of the labels, and succinate alone did not reduce spin labels at all. TMPD (*N,N,N',N'*-tetramethylphenylenediamine, Wurster's blue) also did not reduce spin labels by itself, although it was necessary to keep the concentration of TMPD below 10^{-5} M to avoid interference of its free radical spectrum with that of the spin labels. Mitochondria alone (in the absence of substrate) also had no effect. The complete reducing system of succinate, mitochondria, and TMPD reduced spin labels, either free

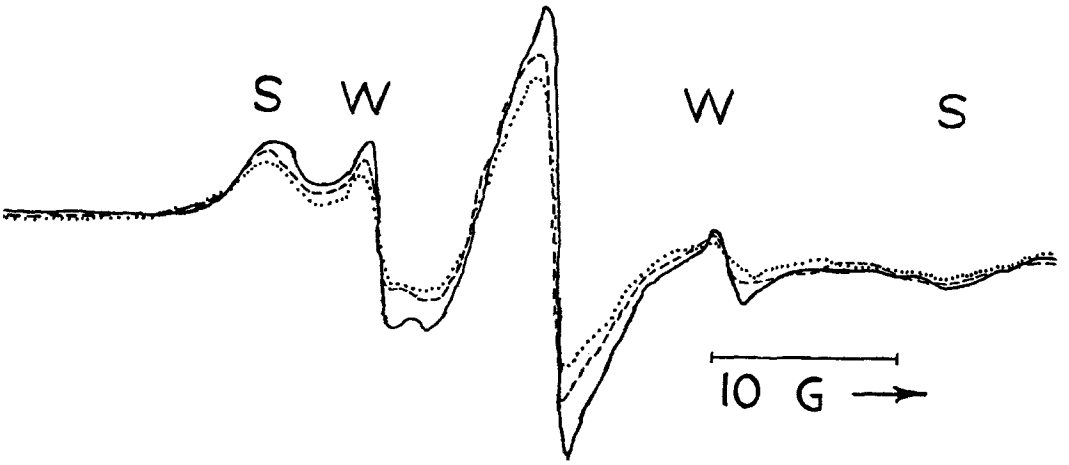

Figure 1. Effects of phospholipid and cytochrome *c* on oxidized soluble cytochrome oxidase. ——, soluble oxidase; – – – –, oxidase plus phospholipid; . . . ., oxidase plus phospholipid plus cytochrome *c*. This oxidase is maleimide labelled; all fractions are oxidized. W, weakly immobilized labels; S, strongly immobilized labels. Instrument gain on phospholipid-cytochrome *c* reduced 20% for clarity.

or bound to a protein, at a rate of approximately 2% per hour. This was the slowest rate obtained for any system capable of reducing cytochrome oxidase except for reduced cytochrome *c*, which was somewhat slower but unsuitable because it could not be added in sufficient concentration.

In order to reduce cytochrome oxidase, the following system was placed in one arm of the anaerobic cell; mitochondria, 0·02 ml of a 50 mg/ml suspension; TMPD, 0·01 ml of a 10^{-4} M solution; and succinate, added last, 0·01 ml of a 2 M solution, pH 6·9. The other arm contained 0·4 ml of cytochrome oxidase at a concentration of 30–50 mg/ml for membraneous and 10–30 mg/ml for soluble. Soluble oxidase was normally supplemented with 0·01 ml of a 1% suspension of phosphatidylethanolamine and 0·01 ml of a 1% solution of cytochrome *c*, to accelerate reduction. Neither the phospholipid nor the cytochrome *c* had any effect on the EPR spectrum of membraneous oxidase, either oxidized or reduced, and did not affect oxidized soluble oxidase (Fig .1). Because of the label reduction problem to be discussed below, it is more difficult to be sure there

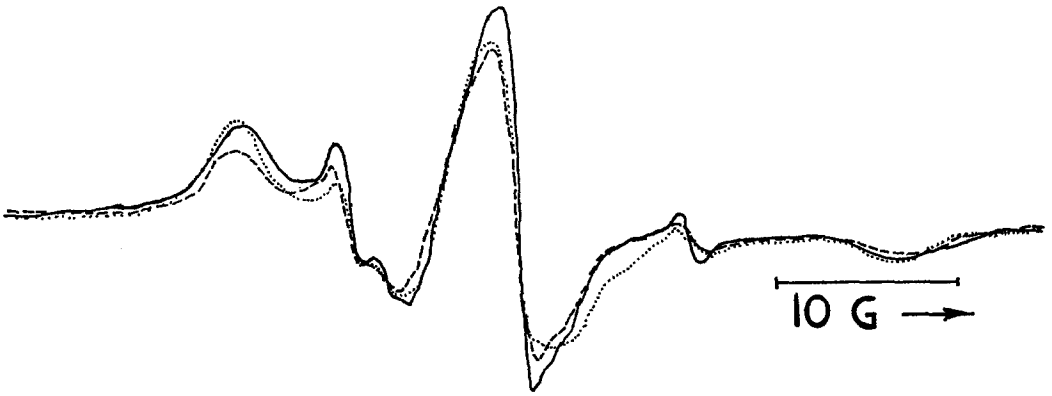

Figure 2. Effect of solubilization on membraneous cytochrome oxidase. The soluble and residue fractions shown are derived from the maleimide labelled membraneous oxidase shown. All fractions are oxidized; amplification has been adjusted to produce spectra of similar amplitude. ——, membraneous; – – – –, soluble; . . . ., residue.

was no effect of cytochrome *c* or phospholipid on reduced soluble oxidase. What can be said is that there was no qualitative change: cytochrome *c* did not affect the *direction* of the observed change, although we cannot demonstrate that it did not affect the extent of that change.

After filling the sidearms, the anaerobic vessel was closed and evacuated until bubbles began to form, at which point it was flushed with nitrogen. This cycle was repeated five times, at about 30 sec per cycle. Finally, the vessel was evacuated as before and closed

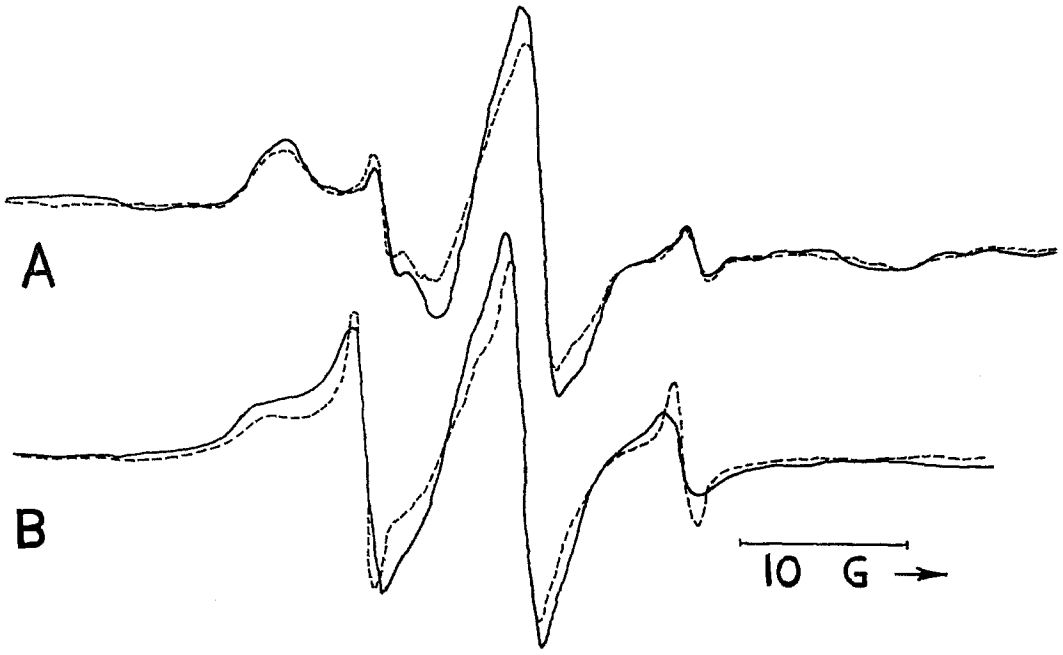

Figure 3. Effect of reduction on membraneous cytochrome oxidase. A, maleimide labelled; B, iodoacetamide labelled. ——, oxidized; – – – –, reduced. The reducing system described in the text was used. The reduced spectra are of fully reduced oxidase; spectra taken at 75% reduction show the same trend. Spectra at the same instrument gain in A, in B, reduced is amplified 20% relative to A.

off, and the oxidase was mixed with the reducing system and allowed to flow into the aqueous EPR cell. Complete reduction required 3 to 5 min for membraneous preparations, and 8 to 15 min for soluble; 75% reduction was achieved in about half these times. As far as we could determine, this variation was caused by the variation in the activity of both the mitochondria and the oxidase. It was impossible to remove all oxygen from the solutions by bubbling with nitrogen beforehand because of frothing of the detergent-containing solutions.

The effect of solubilization on the EPR spectrum of maleimide-labelled cytochrome

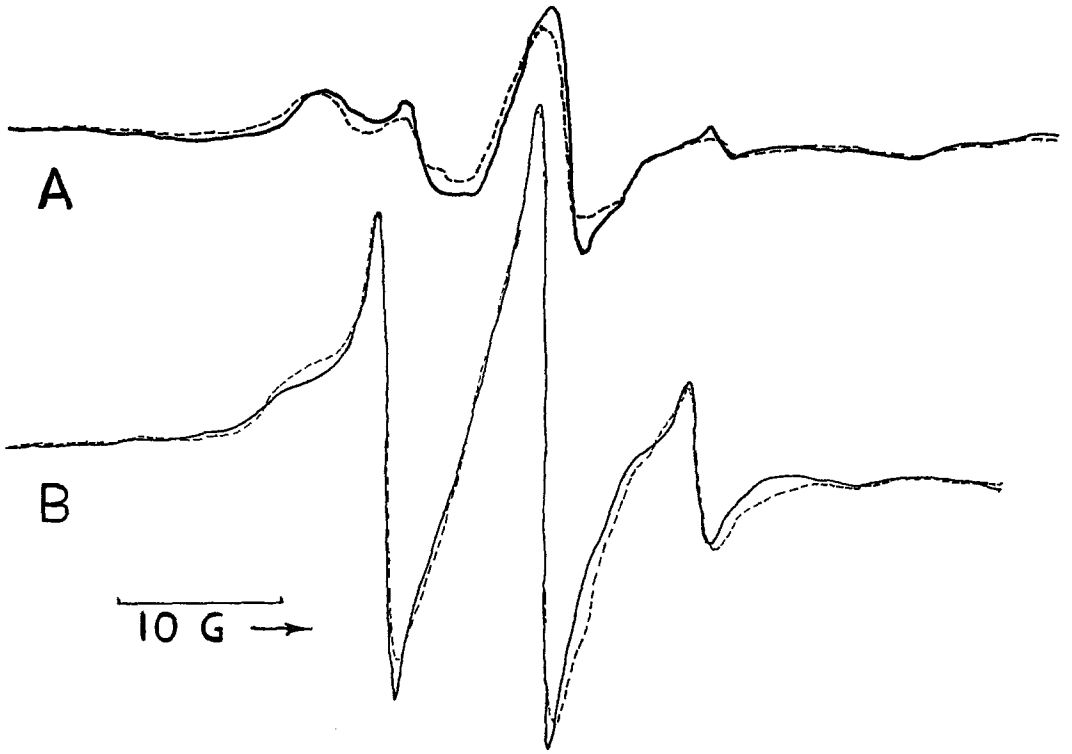

Figure 4. Effect of reduction on soluble cytochrome oxidase. ——, oxidized; – – –, reduced. A, maleimide labelled; B, iodoacetamide labelled. The reducing system described in the text was used, including phospholipid and cytochrome *c*. The spectra shown are fully reduced and were selected for minimum amount of reduction of label. Reduced spectra are amplified 20% (relative to oxidized) in A and 40% in B.

oxidase is shown in Fig. 2. The pattern with iodoacetamide label is the same: the spin label EPR spectra of soluble and membraneous oxidase are virtually identical, and "residue" has relatively less weakly immobilized label, although it contains approximately the same amount of label per milligram of protein as the oxidase fractions. The relative amounts of weakly and strongly immobilized label was somewhat variable from preparation to preparation and seemed to correlate with the amount of lipid removed from the membraneous oxidase before labelling. (However, concentrated lipid extracts from spin labelled membraneous oxidase showed no signal.) Such variation never affected the direction of the changes observed on reduction.

The effect of reduction of membraneous oxidase is shown in Fig. 3. The important

feature is the increase in the amount of weakly immobilized label relative to strongly immobilized.

When these preparations are solubilized, however, the direction of change is reversed with maleimide label and made ambiguous with iodoacetamide label (Fig. 4). Reduced maleimide-labelled soluble cytochrome oxidase has more strongly immobilized label than does its oxidized analog; and there is either no difference, or a small change toward strongly immobilized, between oxidized and reduced iodoacetamide-labelled soluble cytochrome oxidase. These observations are summarized in Table I.

TABLE I. Change in peak ratios upon reduction of cytochrome oxidase

Preparation	Membraneous		Soluble	
label	W/S	W/M	W/S	W/M
Maleimide:				
Oxidized	0·70	0·23	0·85	0·31
Reduced	1·01	0·35	0·58	0·23
Difference	−0·31	−0·12	+0·27	+0·08
(Percent)	−30%	−34%	+32%	+26%
Iodoacetamide:				
Oxidized	1·30	0·57	3·30	0·69
Reduced	1·72	0·73	2·73	0·69
Difference	−0·42	−0·16	+0·57	+0·00
(Percent)	−25%	−22%	+17%	0%

Ratios are taken from Figs. 3 and 4. W/S indicates the ratio of the height above the baseline of the two peaks to the left of the graph (see Fig. 1; a point the same distance to the left from W is taken as S for iodoacetamide spectra). W/M indicates the ratio of the left "W" peak to the large central peak (above the baseline). An increase in either ratio indicates (qualitatively) an increase in immobilized label; a decrease indicates a decrease of the same, or an increase of strongly immobilized label. Difference is oxidized minus reduced; percent is calculated using the larger figure (oxidized or reduced) as base.

Application of this reducing system to either residue or to spin labelled bovine serum albumen produced no conformational changes either in the presence or the absence of detergents, although it did reduce the spin labels attached to these proteins at a rate of 2–4% per hour.

Simultaneously with reduction of the oxidase, the spin labels themselves were being reduced. Typical rates of label reduction are shown in Fig. 5. All membraneous preparations were close to the one shown; the range of variation for soluble preparations was considerably wider. It is noteworthy that the rate with cytochrome oxidase is much greater than the rate in its absence, indicating that cytochrome oxidase may catalyze spin label reduction. This possibility deserves more detailed investigation than we have been able to give it to date.

The possibility that selective reduction of the labels caused the changes in the spin label spectrum described above cannot be entirely eliminated, but two types of evidence

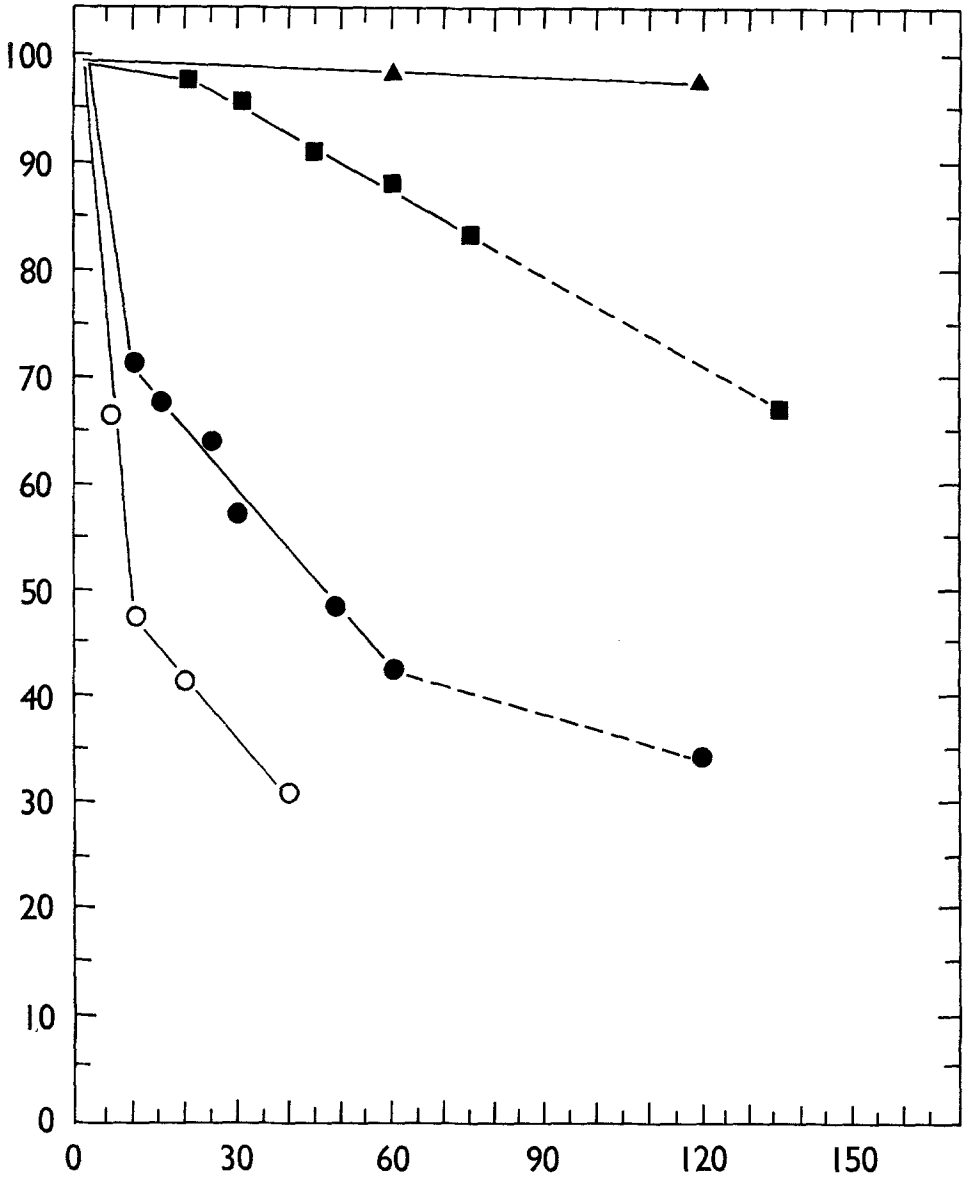

Figure 5. Rate of reduction of spin labels by various systems. ▲, succinate-TMPD-mitochondria reducing system only (see text for composition) operating on unattached spin label; ■, reducing system plus membraneous cytochrome oxidase (label is attached to the cytochrome oxidase); ●, reducing system plus fresh soluble cytochrome oxidase; ○, reducing system plus aged cytochrome oxidase. The amount of label before addition of reducing system, compensated for dilution, is taken as 100%. ——, experimental; – – –, calculated at 20 h rate.

suggest that this is not the case. Reduction of labels without reduction of the oxidase was observed when the cell was left open, or when the succinate concentration was reduced. In these cases the changes in the EPR signal attributed to reduction of the cytochrome oxidase were not observed, although the usual rate of label reduction was maintained.

Figure 6. Long-term reduction of spin labels. ——, soluble oxidase, 1 h after addition of reducing system; – – – –, same solution, 21 h later (gain is 20× higher).

Furthermore, extensive reduction of the labels of both membraneous and soluble oxidase showed only slight preferential reduction of the free signal over a period of 20 h (Fig. 6). While neither of these arguments completely eliminates the possibility that the observed differences between membraneous and soluble oxidase are due to highly specific reduction of the "free" labels of soluble oxidase during the first few minutes after addition of succinate and then only when the cell has been evacuated, we feel that this possibility is rather unlikely.

Discussion

We have observed that there is little change in the EPR spectrum of oxidized spin-labelled cytochrome oxidase when it is solubilized with Triton X-100. On reduction, however, the soluble preparations behave differently from the membraneous ones: in membraneous oxidase, the conformational change that occurs causes more of the spin label to be weakly immobilized, while in soluble oxidase the conformational change that occurs is in some way different, so that the net change in the labels' environment is

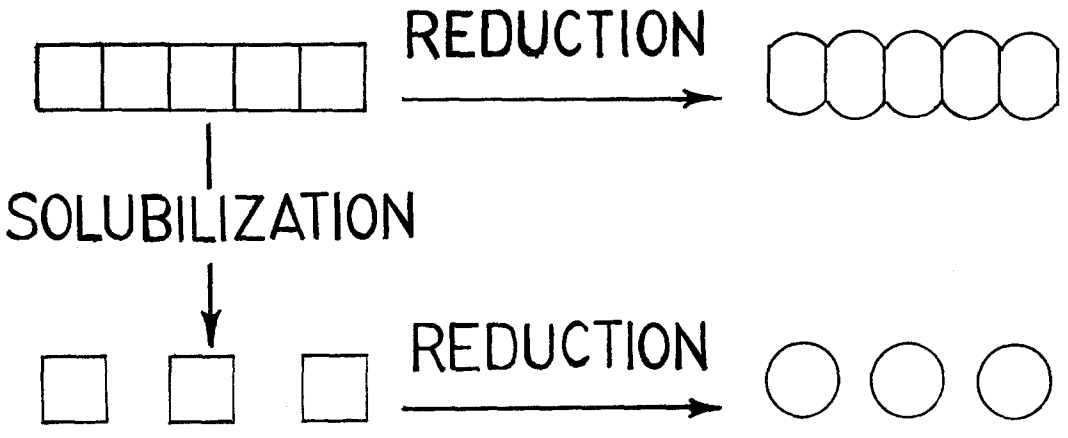

Figure 7. Representation of effects of solubilization and reduction. Upper left, oxidized membraneous oxidase; upper right, reduced membraneous oxidase; lower left, oxidized soluble oxidase; lower right, reduced soluble oxidase.

either zero or in favour of the strongly immobilized fraction. This result is in accord with the results of Myer and King,[1] who showed that aging or desoxycholate change the circular dichroic spectra of reduced cytochrome oxidase much more than they change the spectra of oxidized cytochrome oxidase. The change caused by solubilization also accords well with Jacob's[9] earlier discovery that solubilization abolishes tetrachlorobenzoquinone oxidase activity in cytochrome oxidase.

Our interpretation of these results is that oxidized cytochrome oxidase is in a sense "relaxed", so that separation of the subunits (solubilization) or removal of lipid (solubilization, desoxycholate, and probably aging) does not markedly alter the tertiary structure of the subunit. However, reduction of the oxidase puts it in a state of "strain", so that removal of adjacent subunits, or of lipid, changes the preferred conformation of the reduced state. A purely schematic presentation of this idea is given in Fig. 7.

Both the soluble and membraneous oxidase presumably assume the conformation of lowest energy in both the oxidized and reduced states. There are two possible reasons (not, unfortunately, mutually exclusive) for the differences between the reduced states of the two forms of oxidase. The soluble oxidase may assume a different state when reduced because removal of lipid has made that state more energetically favorable; or it may assume a different state because monomers bound in a membrane are physically prevented from assuming the state that solubilized monomers do because of intersubunit interaction in the membrane. The latter possibility would imply that solubilized cytochrome oxidase is automatically and probably irreversibly uncoupled from energy conservation (as implied by several other authors[16–19]).

This change on solubilization of the conformation of reduced cytochrome oxidase also indicates that there is no necessary reason for the reactions and kinetics observed in the soluble state to be reproducible in the membraneous state or in the mitochondrion.

Acknowledgements

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