Lowered Respiratory Activity in Hypo-osmotically Shocked Mitochondria without Loss of Cytochrome *c*

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Brief exposure of rat liver mitochondria to hypo-osmotic sucrose media caused a decline in the rates of succinate and ascorbate-*NNN'*N'-tetramethyl-*p*-phenylenediamine oxidation without loss of cytochrome *c* when assayed in iso-osmotic media. Lowered respiration rates in mitochondria after brief exposure to hypo-osmotic media may reflect a modification of cytochrome *c* binding.

Siekevitz & Watson (1956) reported that, in mitochondria suspended in 30 mM-sucrose, succinate oxidase activity was lower than that observed in intact mitochondria suspended in 0.44 M-sucrose and that exogenous cytochrome *c* could restore the full succinate oxidase activity. Jacobs & Sanadi (1960*a,b*) showed that cytochrome *c* could be removed from the inner membrane by subjecting mitochondria to hypo-osmotic shock, to rupture the outer membrane, followed by extensive washing in electrolyte solution. MacLennan *et al.* (1966), using frozen and thawed bovine heart mitochondria exposed to hypo-osmotic medium followed by washing with medium containing 150 mM-KCl, showed that cytochrome *c* was removed and that the rates of oxidation of pyruvate plus malate, succinate and ascorbate-*NNN'*N'-tetramethyl-*p*-phenylenediamine were lowered.

Matlib & O'Brien (1976) have shown that cytochrome *c* is released from the inner membrane of mitochondria treated with digitonin to disrupt the outer membrane, the mitochondria being suspended in an iso-osmotic electrolyte medium. Thus removal of cytochrome *c* in ionic media does not require prior hypo-osmotic treatment, as used in the techniques of Jacobs & Sanadi (1960*a,b*).

Streichman & Avi-Dor (1967) have shown that, in mitochondria osmotically shocked in hypo-osmotic sucrose medium and then placed in near-iso-osmotic ionic media, respiratory control was lowered, respiratory activity diminished and cytochrome *c* released from the inner membrane.

The present paper reports the effects of brief hypo-osmotic shock on both succinate and ascorbate-*NNN'*N'-tetramethyl-*p*-phenylenediamine oxidase activities in uncoupled mitochondria. We report a lowering of respiratory activity under conditions in which cytochrome *c* is not removed from the mitochondrial inner membrane.

Experimental

All animals used in this investigation were 3-month-old female rats bred in this laboratory from an original Wistar strain. Liver mitochondria were prepared by the method of Chappell & Hansford (1969), in isolation medium containing 0.25 M-sucrose, 5 mM-Hepes [2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl]ethanesulphonic acid] and 0.5 mM-EGTA [ethanedioxybis-(ethylamine)-*NNN'*N'-tetra-acetic acid], adjusted to pH 7.4 with 1 M-NaOH. Protein concentration was measured by the biuret method (Gornall *et al.*, 1949) after clarification with Triton X-100. Bovine serum albumin was used as a standard. The procedure for brief exposure of mitochondria to hypo-osmotic media was as previously described (Barrett & Horton, 1976), in which the mitochondria (4-6 mg of protein) were added to medium, at 25°C, comprising 3.25 mM-KH₂PO₄/KOH buffer, pH 7.4, and either 13 mM-KCl or 26 mM-sucrose in the stirred oxygen-electrode chamber such that the final volume was 3.08 ml. After 20 s 0.92 ml of 0.5 M-KCl or 1 M-sucrose was added to bring the volume to 4 ml. Rotenone and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were then added such that the final composition of the mixture was either 0.125 M-KCl or 0.25 M-sucrose, and 2.5 mM-KH₂PO₄/KOH buffer, pH 7.4, 1.25 µg of rotenone/ml and 0.25 µM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The succinate oxidase activity was measured after the addition of succinate (2.5 mM), and, in separate experiments, the ascorbate-*NNN'*N'-tetramethyl-*p*-phenylenediamine oxidase activity was measured after the addition of ascorbate (5 mM) and *NNN'*N'-tetramethyl-*p*-phenylenediamine (0.5 mM) to the assay medium supplemented with 1.25 µg of antimycin A/mg of mitochondrial protein. The respiratory control ratios of the untreated mitochondria and mitochondria briefly

exposed to hypo-osmotic media were measured for ADP-stimulated succinate oxidation as previously described (Barrett & Horton, 1976), but in the medium described above for succinate oxidase activity assays.

Exposure of mitochondria to hypo-osmotic media was also performed on a larger scale in which the final volume was 50 ml containing 50 mg of mitochondrial protein. The composition of the media was as described above. The treated suspension in 0.25 M-sucrose or 0.125 M-KCl media was centrifuged at $105\,000g_{av.}$ for $1\frac{1}{2}$ h at 2°C in an MSE Superspeed 50 ultracentrifuge, to sediment the membrane fraction. The adenylate kinase activities in the supernatant fractions and in the untreated mitochondria were measured by the method of Schnaitman & Greenawalt (1968). The bulk of the supernatant was concentrated by vacuum dialysis to about one-tenth of the original volume. The dithionite-reduced-minus-ferricyanide-oxidized absorption difference spectrum was recorded with a Perkin Elmer 356 spectrophotometer. Cytochrome *c* content was estimated by using the absorption difference of the wavelength pair 550/540 nm for reduced-minus-oxidized cytochrome *c* (Chance & Williams, 1956). The total cytochrome *c* content of untreated mitochondria was determined in mitochondria solubilized with Triton X-100, by the method of Williams (1964).

ADP, succinic acid and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine were obtained from Boehringer Corporation (London), Lewes, East Sussex, U.K. Rotenone, antimycin A, EGTA, cytochrome *c* (type VI) and L-ascorbate were from Sigma (London) Chemical Co., Kingston-upon-

Thames, Surrey, U.K. *NNN'*-Tetramethyl-*p*-phenylenediamine was from BDH Chemicals, Poole, Dorset, U.K. Hepes was from Hopkin and Williams, Romford, Essex, U.K. All other reagents were of A.R. grade.

Results

The effects of brief (20 s) exposure to hypo-osmotic KCl and sucrose media on the uncoupled succinate and ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine rates of oxidation are shown in Table 1, together with the rates of oxygen uptake with these substrates in untreated mitochondria suspended in iso-osmotic sucrose and KCl media. In both media the effect of brief exposure to hypo-osmotic conditions is to lower the rate of substrate oxidation. The extent of the change is greater in KCl medium than in sucrose medium. MacLennan *et al.* (1966) and Streichman & Avi-Dor (1967) have shown that, after hypo-osmotic shock and subsequent resuspension in ionic media, which results in a loss of cytochrome *c*, a decrease in the rates of oxidation of a number of substrates, when assayed in predominantly ionic medium, occurred. The experiments now reported show a decrease in rates of oxidation under both ionic and non-ionic assay conditions. The loss of activity in ionic (KCl) media was about four times as great as the loss of activity in non-ionic (sucrose) media. The amount of cytochrome *c* lost during brief exposure of mitochondria to hypo-osmotic media was therefore investigated.

After brief exposure of mitochondria (50 mg of protein) to hypo-osmotic media, the supernatant fraction

Table 1. *Effect of brief exposure to hypo-osmotic media on the succinate and ascorbate-NNN'-tetramethyl-p-phenylenediamine oxidase activities of rat liver mitochondria*

The details of brief (20 s) exposure to hypo-osmotic media are as described in the Experimental section. Oxidase activities were measured polarographically at 25°C in medium containing 2.5 mM- KH_2PO_4 /KOH buffer, pH 7.4, 1.25 μg of rotenone/ml, 0.25 μM -carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine and either 0.125 M-KCl or 0.25 M-sucrose as stated. The succinate concentration was 2.5 mM for succinate oxidase assays and for the ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine oxidase assays 5 mM-ascorbate and 0.5 mM-*NNN'*-tetramethyl-*p*-phenylenediamine were used in assay medium supplemented with antimycin A (1.25 μg /mg of mitochondrial protein). Mitochondria were used at a final concentration of 1–1.5 mg of protein/ml. Liver mitochondria, separately isolated from five rats, were assayed in triplicate, and the results for each batch of mitochondria were averaged. The final results are the means $\pm$ S.D. of the five separate experiments.

Suspension medium	Substrate	Oxygen uptake rate (ng-atoms of O/min per mg)		Loss of activity after treatment (%)
		Untreated mitochondria	Treated mitochondria	
KCl	Succinate	143 ± 10.2	41 ± 10.8	70
KCl	Ascorbate- <i>NNN'</i> -tetramethyl- <i>p</i> -phenylenediamine	199 ± 21.1	62 ± 16.1	
Sucrose	Succinate	101 ± 16.8	82 ± 10.2	18
Sucrose	Ascorbate- <i>NNN'</i> -tetramethyl- <i>p</i> -phenylenediamine	174 ± 30.1	142 ± 10.6	

was assayed for adenylate kinase activity (a marker enzyme for the intermembrane space). The remainder of the supernatant was concentrated and assayed for cytochrome *c* as described above. Brief exposure to both hypo-osmotic sucrose and KCl media was found to cause the release of $90 \pm 2\%$ ($n = 3$) of the total adenylate kinase activity into the supernatant. Brief exposure to hypo-osmotic sucrose medium released some cytochrome *b_s* from the outer membrane, but cytochrome *c* was not detected by the spectrophotometric method used. If any cytochrome *c* were present in the supernatant fraction it would represent much less than 0.5% of the total cytochrome *c* content of the mitochondria. In contrast, and as expected, KCl medium caused the release of cytochrome *c* into the supernatant fraction. It was found that $21 \pm 8.5\%$ ($n = 3$) of the total cytochrome *c* was released into the supernatant fraction. Increasing the volume of the system from 4 ml, as used in the oxidase assays, to 50 ml did not affect the oxidase activities as determined in control experiments.

Electron microscopy of mitochondria showed that, after brief exposure to hypo-osmotic sucrose or KCl media and the return to iso-osmotic sucrose or KCl media, the outer membranes were swollen or burst and the inner membranes were contracted inwards from the outer membranes. The respiratory control ratios of ADP-stimulated succinate oxidation in mitochondria after brief exposure to hypo-osmotic conditions were 2.4 ± 0.1 ($n = 4$) in sucrose media and 2.2 ± 0.3 ($n = 4$) in KCl media. The corresponding values for untreated mitochondria were 4.0 ± 0.1 ($n = 4$) and 4.2 ± 0.15 ($n = 4$) respectively.

In similar experiments to those reported above, the inclusion of cytochrome *c* in the hypo-osmotic treatment medium at a final concentration of $10 \mu\text{M}$ in the 4 ml assay mixture increased the succinate oxidase activity in uncoupled mitochondria but did not affect the activity in mitochondria not subjected to hypo-osmotic exposure. In KCl medium the uncoupled succinate oxidase activity was increased from 41 to 119 ng-atoms of O/min per mg, whereas in sucrose medium the activity was increased from 82 to 91 ng-atoms of O/min per mg. These trends are slightly different in extent from those observed by Siekevitz & Watson (1956), who used mitochondria suspended in 30 mM-sucrose.

Discussion

The loss of cytochrome *c* in KCl media and the corresponding loss of respiratory activity (Table 1) and respiratory control (see the Results section) are consistent with the experimental findings of Streichman & Avi-Dor (1967), although in their experiments the details of the osmotic-shock procedure are slightly different from those used in the present investigation.

The results (Table 1) indicate that the loss of succinate oxidase and ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine oxidase activities as a result of brief exposure to hypo-osmotic media are not solely attributable to the loss of cytochrome *c*, since the loss of the oxidase activities is not stoichiometric with the loss of cytochrome *c*, and in fact virtually no cytochrome *c* is lost in sucrose medium. That the lowering of oxidase activity after exposure to sucrose media may have been due to cytochrome *c* being leached from the inner membrane during the period of the oxidase assays is discounted. The time-period between the end of the large-scale exposure to hypo-osmotic sucrose and the commencement of centrifugation (about 5 min) was about the same period as that required for the oxidase assays. If any cytochrome *c* were to be leached out during this short assay period, it would be expected to appear in the supernatant fraction after centrifugation, but none was detected. In contrast, the near-complete loss of intermembrane-space components, as typified by the marker, adenylate kinase, indicates extensive rupture of the outer membrane.

The results given in the present paper extend the observations made by Streichman & Avi-Dor (1967) in that brief exposure of mitochondria to hypo-osmotic sucrose followed by a return to an iso-osmotic state by the addition of sucrose does not result in a loss of cytochrome *c*. However, this treatment does result in a loss of respiratory activity, as measured with the succinate oxidase and ascorbate-*NNN'*-tetramethyl-*p*-phenylenediamine oxidase in uncoupled mitochondria. The loss of respiratory activity under these conditions suggests a modification of cytochrome *c* binding. The relative decreases of the two oxidase activities are identical (Table 1), indicating that any modification to the respiratory chain as a result of the treatment probably occurs at or after the *NNN'*-tetramethyl-*p*-phenylenediamine-binding site (cytochrome *c₁*). Cytochrome *c* is the most labile component of the respiratory chain between cytochromes *c₁* and *a₃*, and consequently is perhaps the most likely component for modification of its binding properties.

The partial restoration of succinate oxidase activity, after brief exposure to hypo-osmotic sucrose medium, by the inclusion of exogenous cytochrome *c* in the hypo-osmotic exposure and iso-osmotic assay media has been demonstrated in this present study. No cytochrome *c* was released during the treatment in sucrose medium. The exogenous cytochrome *c* may act by bridging the modified binding sites for endogenous cytochrome *c* on the inner membrane and so allow an increased electron flux.

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