THE ENERGY-LINKED REDUCTION OF NADP+ BY SUCCINATE, AND ITS RELATIONSHIP TO CHOLESTEROL SIDE-CHAIN CLEAVAGE

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1. Introduction

In a previous publication [1] a mitochondrial preparation derived from porcine corpora lutea was decribed. This preparation contained a cytochrome P450 dependent mixed function oxidase which catalysed cholesterol side chain cleavage (SCC) and which was active only if supplied with a suitable electron donor. When succinate was used as the sole source of electrons, the SCC reaction was energy dependent. The study of cytochrome P450 mediated steroid hydroxylation reactions in adrenocortical mitochondria, which can also be supported by succinate oxidation, has lead several groups of workers to suggest that reducing equivalents are transferred from succinate to the steroid hydroxylation site via two endergonic processes. The first energy requiring process is reversed electron flow between the succinate dehydrogenase flavoprotein and NAD^+ and the second, a pyridine nucleotide transhydrogenation from NADH to $NADP^+$; subsequently the NADPH–cytochrome P450 reductase is reduced [2–4].

In contrast to reports pertaining to adrenocortical preparations, we demonstrated that in the porcine corpus luteum the pyridine nucleotide transhydrogenase associated with SCC is not necessarily energy-linked [1]. The work reported here shows that the electron flow pathway from succinate to the NADPH–cytochrome P450 reductase in our porcine preparations is also different from that described in the adrenal cortex; our results indicate that although the reduction of $NADP^+$ by succinate is energy dependent, it does not proceed via NAD^+.

2. Materials and methods

[4-^{14}C] cholesterol (58 μCi/mg) was obtained from the Radiochemical Centre, Amersham. Succinate, ATP, NAD^+, $NADP^+$, rotenone, amytal, antimycin, DNP and bovine serum albumin (fatty acid free) (BSA) were obtained from Sigma, London. [4-^{14}C] cholesterol, rotenone and DNP were purified before use as described previously [1]. Potassium cyanide was obtained from British Drug Houses (Poole, England) and made up as a neutral solution immediately before use. All other reagents or solvents were of analytical grade.

The preparation of our mitochondrial fraction from porcine ovarian corpora lutea, and the SCC assay were described in an earlier paper [1]. The reaction was carried out in a buffered medium consisting of 200 mM sucrose, 25 mM Tris-HCl pH 7.4, 10 mM potassium phosphate pH 7.4, 20 mM KCl, 5 mM $MgCl_2$, 0.2 mM tetra sodium EDTA and 1% (w/v) BSA. Succinate and inhbitors were added as required. The SCC reaction, which was started by the addition of 1 μg of [4-^{14}C] cholesterol (100,000 cpm) to whole mitochondria (about 3 mg protein) suspended in 1 ml of the above medium, proceeded for 1 hr at 37° in an atmosphere of air. The reaction was stopped by adding 1 ml of methanol containing 4 μg of cholesterol, 2 μg pregnenolone and 2 μg progesterone. The steroids were extracted, and separated by TLC on silica gel G as before [1]. SCC activity was expressed as the percent conversion of [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone in 1 hr.

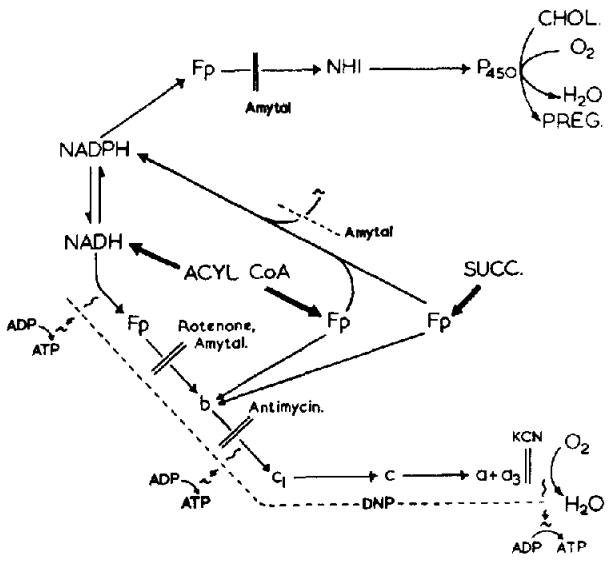

Fig. 1. A schematic representation of the postulated interaction between the cytochrome oxidase chain and the P450 steroidogenic chain in porcine luteal mitochondria. The cytochromes are designated by their letter nomenclature. Sites of energy conservation and utilization are shown, and ~ represents a high energy intermediate in ATP formation. Sites of inhibition of electron transfer are shown at //. Solid arrows (→) indicate direction of electron flow. Uncoupling of energy conservation reactions from electron flow is depicted by a broken line (- - - -). NHI = non-haem iron protein, chol=cholesterol, preg=pregnenolone, and succ=succinate.

Polarographic determinations of oxygen uptake were carried out at 30° as previously [1, 5] with whole mitochondria (approx. 10 mg protein) suspended in 3 mls of the above medium using a Clark oxygen electrode. ADP was added to a final concentration of 1 mM.

The reduction of $NAD(P)^+$ by luteal mitochondria in the presence of succinate was observed spectrophotometrically at 340 nm with a Shimadzu split beam recording spectrophotometer (Model MPS-50L). The reaction was carried out in 2.9 ml of the medium described above, to which was added 0.1 ml of a mitochondrial preparation, which had been sonicated in 1 ml lots for 20 sec at 2 amp, succinate to a final concentration of 2 mM, ATP to 2 mM and $NAD(P)^+$ to 500 μM in that order. The reaction was recorded continuously between each addition.

3. Results and discussion

Ovarian mitochondria are specialized organelles the function of which is not only energy production, but also steroid hydroxylation. It is evident that these two functions are interrelated and therefore it should not be surprising if the classical pathways worked out for liver and heart mitochondria are not the same in these steroidogenic particles.

When supported by succinate or free fatty acids (FFA) SCC can proceed only if energy is available [1, 6]. Fig. 1 illustrates a proposed scheme of electron flow in porcine luteal mitochondria, from succinate (and FFA) to cytochrome oxidase via the respiratory chain, and to the cytochrome P450 containing cholesterol hydroxylation site. Some of our previous work which relates to this scheme has already been reported [1, 6]. The experimental results which are discussed below satisfy two major tenets of this hypothesis. First, they demonstrate that, in the absence of added ATP, it is necessary to produce energy for SCC via the respiratory pathway when succinate is the source of reducing potential for this reaction; and second, they show that electrons from succinate pass to $NADP^+$ by a route which does not include reversed electron flow to NAD^+. The reduction of $NADP^+$ by succinate is energy dependent.

3.1. *Demonstration of energy requirement for succinate-supported SCC*

Polarographic investigations established that when succinate was present at a concentration of 5 mM, 1 mM KCN reduced the oxygen uptake by intact luteal mitochondria in "state 4" (i.e. in the absence of added ADP) from 8 to < 1 nmole O_2/mg protein/min and antimycin at a concentration of 330 ng/mg of mitochondrial protein reduced it from 8 to 2 nmole O_2/mg protein/min. It was shown previously that at these concentrations neither KCN nor antimycin inhibits SCC supported by NAD(P)H [1] and yet it can be seen in table 1 that both these substances virtually abolish SCC when it is supported by succinate Table 1 shows that the inhibition of SCC by antimycin and KCN is reversed by ATP. This suggests that energy is utilised to reduce the NADPH–cytochrome P450 reductase by succinate, and that in the absence of respiratory inhibitors this energy is derived from succinate oxidation via cytochrome oxidase.

Table 1
The effect of various inhibitors on succinate-supported cholesterol side-chain cleavage.

Substrate and other compounds present	Per cent conversion of [4-^{14}C] cholesterol to [4-^{14}C] steroid products		
	(a) [4-^{14}C] Pregnenolone	(b) [4-^{14}C] Progesterone	(c) Total conversion
No additions	0.1	0.3	0.4
Succ.	4.5	14.3	18.8
Succ. + rotenone	5.6	17.9	23.5
Succ. + amytal	3.0	7.7	10.7
Succ. + antimycin	1.2	1.6	2.8
Succ. + rotenone + ATP	6.5	12.7	19.1
Succ. + amytal + ATP	4.5	6.4	10.9
Succ. + antimycin + ATP	14.1	8.6	22.7
Succ. + ATP	6.4	14.5	20.9
ATP	1.7	4.5	6.2
ATP + rotenone	4.1	4.2	8.3
ATP + amytal	3.9	3.7	7.6
ATP + antimycin	0.3	0.8	1.1
Succ. + cyanide	0.2	0.5	0.7
Succ. + cyanide + ATP	11.7	7.5	19.2

Porcine luteal mitochondrial preparations were incubated in an isotonic medium for 1 hr, and cholesterol side-chain cleavage activity determined, as described in the text. Final concentrations of the compounds listed above were as follows: succinate, 5 mM; ATP, 2 mM; rotenone, 10 μM; amytal, 1.8 mM; cyanide, 1 mM; DNP, 100 μM; antimycin, 330 ng per mg of mitochondria protein. Each incubation contained 3 mg of mitochondrial protein.

Since ADP was not present in the incubation used to measure SCC the energy derived from respiration and used to drive electrons from succinate to the cytochrome P450 chain is unlikely to be in the form of ATP, but rather as a high energy intermediate $x \sim y$ or $x \sim p$.

3.2. *Evidence against the involvement of NAD dependent reversed electron flow*

It was shown that two inhibitors, rotenone and amytal, which block the respiratory chain between NADH and cytochrome *b* in bovine heart mitochondria [7] also prevent oxygen uptake by sonicated luteal mitochondria when NADH is added. They do not affect oxygen uptake by preparations of porcine luteal mitochondria when succinate is the substrate. Although rotenone can be made specific for a site on the oxygen side of NADH dehydrogenase flavo protein [NADH (acceptor) oxidoreductase (E.C. 1.6.99.3)] by using it at suitable concentrations in the presence of BSA [7, 8], amytal is known to attack other areas as well; e.g. those concerned with the formation and hydrolysis of ATP [9, 11]. If electrons from succinate reach cytochrome P450 via NAD one would expect SCC to be inhibited by these substances which prevent electron transfer from cytochrome *b* to NAD when succinate is the energy source.

It can be seen from table 1 that 10 μM rotenone does not inhibit succinate supported SCC at all (although at 25 μM it does in bovine corpus luteum [12]), while amytal inhibits the cleavage by 50%. This effect of amytal could not be reversed with ATP. Subsequently it was shown that amytal inhibition was occurring on the NADPH–cytochrome P450 chain: NADPH supported SCC was inhibited by 50% when amytal was added to the incubation.

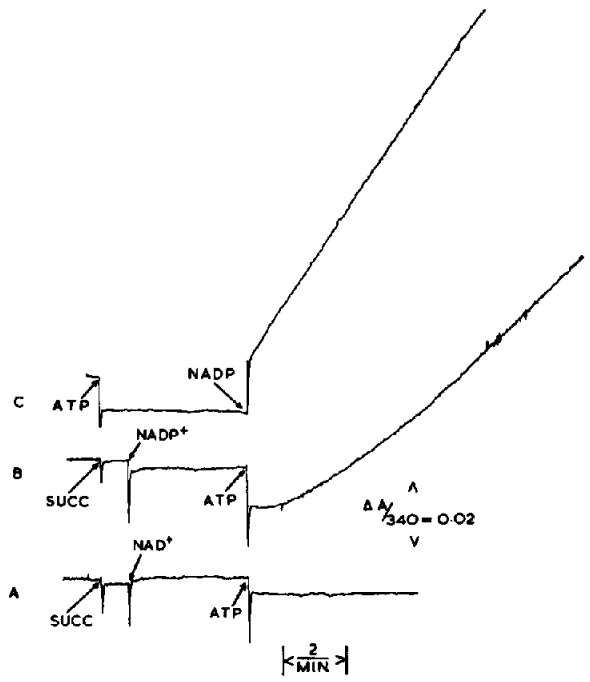

Fig. 2. Energy dependent reduction of $NADP^+$ by succinate. Reaction was carried out in 3 ml buffered medium described in the text, to which was added sonicated mitochondria (0.2 mg protein), 6 μmole succinate, 6 μmole ATP and 9 μmole $NAD(P)^+$ as indicated on the traces. The reduction of NAD(P) was read at 340 nm against a blank which contained all components except succinate.

Uzgiris et al. [12] have also observed this effect of amytal on the cytochrome P450 chain in bovine corpus luteum. It appears that electrons from succinate do not reach the site of cholesterol hydroxylation via reversed electron flow to NAD.

3.3. *The reduction of $NADP^+$ by succinate*

More direct information about the electron transfer pathway between succinate and the NADPH–cytochrome P450 reductase was obtained by spectrophotometric experiments like that described in fig. 2. This depicts the reduction of pyridine nucleotides by succinate, catalysed by preparations of sonicated porcine luteal mitochondria. The results are complementary to those of table 1 and lend further weight to the hypothesis outlined in fig. 1.

The significant points of fig. 2 are firstly that $NADP^+$ was reduced far more rapidly by succinate than was NAD^+, and secondly that the reduction of $NADP^+$ was strictly energy dependent. There was a characteristic lag period (fig. 2, B) if the reaction was initiated by the addition of ATP: this lag was not observed if the sonicated mitochondria were preincubated with ATP, and the reaction started by adding $NADP^+$. This lends support to our earlier observation that a high energy intermediate is involved in this electron transfer rather than ATP per se.

4. Conclusions

Evidence has been presented that the energy-linked electron flow from succinate to the site of SCC does not involve classical reversed electron flow to NAD^+, but proceeds via $NADP^+$. Succinate-supported SCC is not inhibited by rotenone, although it is inhibited 50% by amytal. This inhibition can be explained by the direct effect of amytal on the NADPH supported SCC reaction. These data are compatible with an hypothesis which postulates a direct link between the flavoprotein dependent respiratory dehydrogenases (e.g. succinate dehydrogenase and acyl CoA dehydrogenases) and the NADPH–cytochrome P450 reductase associated with cholesterol side chain cleavage.

Acknowledgements

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