OXALOACETATE AND ADENOSINETRIPHOSPHATE LEVELS DURING INHIBITION AND ACTIVATION OF SUCCINATE OXIDATION

Peter Schollmeyer and Martin Klingenberg
Physiologisch-Chemisches Institut der Universität Marburg

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It had been concluded by some authors, that treatments effecting an inhibition of succinate oxidation increase the level of oxaloacetate, which is known to be a strong inhibitor of the succinate oxidation. However it was difficult to explain on this basis why an inhibition of succinate oxidation by added oxaloacetate could be relieved by ATP (Pardee and Potter 1948), although ATP was found not to remove the oxaloacetate (Tyler 1955). More recently it has been proposed by Azzone and Ernster (1960), on the basis of experiments with arsenate pre-treated liver mitochondria, that the reactivation by ATP demonstrates an energy requirement of succinate oxidation.

As reported in the preceding communication, the succinate oxidation by pigeon breast muscle mitochondria can easily be brought into a state of nearly complete inhibition and subsequently be fully restored by the addition of several substances. In view of current theories on these phenomena, we determined the levels of oxaloacetate and ATP in inhibited and activated states of succinate oxidation. Accurate values on the oxaloacetate level could be obtained by applying sensitive spectrophotometry to the specific method of oxaloacetate determination with malate dehydrogenase. This enables us to detect oxaloacetate down to a concentration of 5×10^{-8} M. In addition phosphoenolpyruvate was measured as a possible reaction product of the oxaloacetate.

Results. The levels of oxaloacetate and phosphoenolpyruvate as given in table 1 have been calculated in terms of concentrations in the mitochondrial suspensions. These values are

obtained in the steady state of activated respiration. For comparison the values of the respiratory activities from the preceding communication (Klingenberg and Schollmeyer 1960), are included in the table.

In most of the experiments oxaloacetate in the range of 10 to 20 μM could be measured. However in a freshly incubated suspension only about 1 μM oxaloacetate is present. When succinate is now added the oxaloacetate level rapidly increases to 15 μM. It is to be noted, that at this stage the oxidation of succinate is not yet inhibited. The same level of oxaloacetate is generated during the pre-incubation period of 15 min., before the addition of succinate. The level of oxaloacetate does not increase further after the addition of succinate, the oxidation of which is now largely inhibited.

The activation of the succinate oxidation by ATP, phosphate or albumin addition does not much change the oxaloacetate level. Characteristic changes of the oxaloacetate concentration are observed when substrates are added to activate the succinate oxidation. Malate nearly doubles the amount of oxaloacetate, presumably as a direct result of the malate oxidation. Ketoglutarate has no effect. On addition of glutamate or cysteine sulfinate the oxaloacetate level decreases below 1 μM. This indicates that the transamination from amino-acids to oxaloacetate is very effective in these mitochondria. In contrast pyruvate is not able to remove oxaloacetate at a rate which would lower significantly the oxaloacetate level.

In principle similar changes of oxaloacetate concentration are found in the respective experiments conducted in the presence of dinitrophenol. It may be recalled however that the uncoupling of oxidative phosphorylation by dinitrophenol largely abolishes the activation of succinate oxidation by most substances, with the exception of the activation by ketoglutarate.

The level of the mitochondrial ATP in the states of inhibition and activation of succinate oxidation has also been measured on extracts by enzymatic analysis. The results are given as the ratio of the ATP to the total amount of adenine

Comparison between the succinate linked respiration and the concentrations of oxaloacetate, phosphoenolpyruvate and ATP in pigeon muscle mitochondria [1]

Additions	Respiration[2]	Oxal-acetate	Phospho-enolpyr.	$\frac{ATP}{\Sigma AP}$[3]
After 2 min. preincubation		uM	uM	
—	0.27	1.0	0	0.48
Succinate	0.95	12.6	0	0.43
After 15 min. preincubation				
—	0.05	13 to 26	0.10	0.10
Succinate	0.06	15.8	0.20	0.09
" + ATP	1.30	15.7	0.70	—
" + Phosphate	1.10	17.7	1.20	0.24
" + Albumin	1.05	17.2	1.10	0.15
" + Malate	1.05	32.5	2.70	0.25
" + Ketoglutarate	1.00	19.5	1.00	0.23
" + Pyruvate	1.20	15.0	—	0.24
" + Glutamate	1.30	0.8	1.20	0.29
" + Cysteine-sulfinate	1.15	0.6	1.00	0.20
After 5 min. preincubation with 0.1 mM dinitrophenol				
Succinate	0.04	15.0	1.45	0.06
" + ATP	0.45	21.0	0.45	—
" + Phosphate	0.10	14.1	0.90	0.10
" + Malate	0.35	31.5	1.00	0.09
" + Pyruvate	0.25	15.0	—	0.09
" + Ketoglutarate	1.20	12.8	0.80	0.17
" + Glutamate	0.90	1.5	1.10	0.20
" + Cysteine-sulfinate	0.35	0.4	0.85	—

1) Conditions cf. Klingenberg and Schollmeyer (1960)

2) $\frac{\mu atom\ O_2/2}{sec\ g\ protein}$

3) $\Sigma AP = ATP + ADP + AMP = 7,4$ to $8,6\ \mu Mol/g$ protein

nucleotide measured. This may be called a "degree of phosphorylation" of the adenine nucleotide system. The total amount

of adenine nucleotide varies between 7.4 and 8.6 µMol/g protein, which corresponds to a concentration of about 45 µMol in the suspension of the mitochondria.

Shortly after incubation about 50 % of the adenine nucleotides exist as ATP. This ratio is lowered to between 9 and 15 % during the pre-incubation period of 15 min. It is not increased by the further addition of succinate, the oxidation of which is now inhibited.

In all instances of reactivated respiration the level of endogenous ATP reaches between 20 to 30 %. It remains below 10 % when the activation is suppressed in the presence of dinitrophenol. However with ketoglutarate or glutamate, also in the presence of dinitrophenol, an increase of the ATP level can be observed in accordance with the activation of succinate oxidation by these substrates.

It is to be noted, that after 15 min. pre-incubation ATP does not reach the original level which can be observed shortly after incubation. Possibly a part of the adenine nucleotide becomes unavailable to oxidative phosphorylation during the pre-incubation. As an alternative explanation could be offered, that an increased ATPase activity decreases the steady state ATP level.

Discussion. In many of the reported experiments oxaloacetate is present at a level sufficiently high to inhibit the succinate oxidation, particularly if one considers that the oxaloacetate concentration inside of the mitochondria could be still much higher. The activation of the succinate oxidation by ATP, which is not accompanied by a decrease of the oxaloacetate level, could then be interpreted, for example following Pardee and Potter (1948) and Tyler (1955), that oxaloacetate has been transformed by ATP to a noninhibitory state. This explanation would have to be extended to the activation found on addition of phosphate, albumin or DPN linked substrates. In fact, in all these cases of activation the level of the endogenous ATP is increased. However, it is difficult to reconcile with this concept that the respiration is not reactivated when, in the presence of dinitrophenol, oxaloacetate is nearly completely

removed by cysteine sulfinate. In this context we might recall previously conducted experiments on liver mitochondria (Azzone, Ernster and Klingenberg 1960) where in an inhibited state of the succinate oxidation the concentration of oxaloacetate was found to be within the limits of accuracy of the measurements, i.e. 5×10^{-8} M. It should be added however, that in the pretreated liver mitochondria with a very low level of oxaloacetate, succinate oxidation could not be inhibited to such an extent as in pigeon breast muscle mitochondria, where a high level of oxaloacetate is observed.

We may summarize that the total results do not support the concept of an inhibition by oxaloacetate. But the results cannot exclude this mechanism. For example, it is conceivable that the oxaloacetate concentration is high at the inhibition site and still very low as an overall concentration in the suspension of the mitochondria. On the other hand, the level of ATP is elevated in all cases of an activated succinate oxidation. This result is in agreement with an energy requirement for the succinate oxidation as proposed by Azzone and Ernster below a certain level.

Attention may be directed to the finding, that dinitrophenol inhibits the activation of the succinate oxidation by ATP to about 60 to 70 % but does not inhibit the activation by ketoglutarate and also by glutamate. It is known and confirmed in our experiments by ATP analysis that dinitrophenol does not prevent the substrate phosphorylation of ketoglutarate oxidation. The experiment shows further that dinitrophenol also does not prevent ATP formed by substrate phosphorylation from activating succinate oxidation.

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