PALMITOYLCARNITINE AND TRICARBOXYLIC-ACID-CYCLE OXIDATIONS

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1. Introduction

Fatty acids in cellular metabolism exist in two activated forms, as acyl-CoA, and as acyl-carnitine. In case of the long chain acyl-derivatives, both types of compounds exhibit numerous effects on various enzymatic reactions [1–7] and therefore have been attributed to exert metabolic control under conditions of enhanced fatty acid oxidation. According to Garland and his group [8–10] the rapid oxidation of palmitoylcarnitine (PC) by liver mitochondria produces a high intramitochondrial energy level, causing an inhibition of citrate synthesis and thus stimulating ketogenesis. Jangaard [11] has reported that with isolated citrate synthase ATP is a competitive inhibitor with respect to oxaloacetate. However, recent studies of Williamson et al. [12–14] have given considerable evidence that the intramitochondrial availability of malate, or oxaloacetate resp., is more important rather than the ATP level for control of citrate synthase in liver. In a previous study Portenhauser and Schäfer [15] have reported that besides the mediated influence of PC on citrate formation a direct effect on the TCA-cycle may exist, by means of a modification of succinate dehydrogenase activity. In the present experiments we have demonstrated that the rate of oxidation of succinate depends on the concentration of PC, present in coupled liver mitochondria, either causing an activation, or an inhibition of respiration.

2. Materials and methods

Livers were obtained from male Wistar rats; mitochondria were isolated in a medium containing 0.3 M sucrose, 1 mM triethanolamine, 0.1 mM EDTA, pH 7.2, as described elsewhere [16]. Protein was measured by a modified Biuret method [17]. Incubations were carried out in a medium containing 0.25 M sucrose, 5 mM K_2HPO_4, 10 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM triethanolamine, pH 7.2 at 25°C; oxygen consumption was measured polarographically with a Clark-type microelectrode in reaction mixtures (2.85 ml) containing approx. 5 mg mitochondrial protein. Redox behaviour of cytochrome *c* was followed by means of a Phoenix Dual wavelength Spectrophotometer at 550–540 nm. Fluorescence of pyridine nucleotides was measured according to the method of Estabrook and Maitra [18]. Deproteinization of samples and fluorimetric enzymatic assay were performed as described previously [16]. Mitochondria suspensions were hypotonically diluted to a protein content of approx. 4.5 mg/ml and succinate dehydrogenase activity was measured as described in ref. [16]. Further details are given in the figures of individual experiments.

3. Results and discussion

The respiration of liver mitochondria with succinate in state 4 [19] (ADP rate-limiting) is gradually increased with increasing concentrations of PC, as demonstrated in fig. 1. The experiment was carried out in the presence of rotenone, abolishing total oxidation of PC itself. As demonstrated by the double-logarithmic regression curves plotted in fig. 1, only L(−)PC causes an activation, whereas the D(+)-isomer has no effect. At high concentrations both optical antipodes

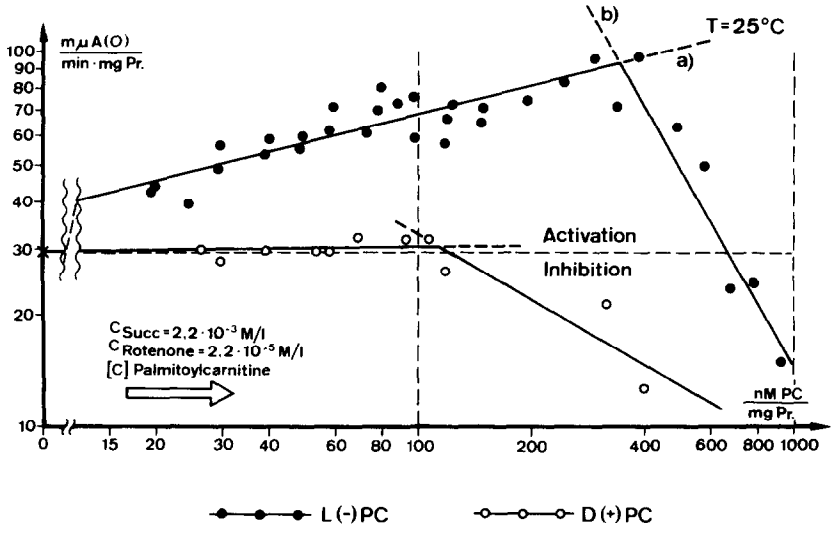

Fig. 1. Influence of L(−)- and D(+)-palmitoylcarnitine on succinate dependent respiration of liver mitochondria in state 4 [19], in presence of rotenone 2.2×10^{-5} M; succinate 2.2×10^{-3} M; for other conditions see "methods". The correlation curves were computed from the individual measurements with L(−)PC (solid circles) and D(+)PC (open circles).

lead to an inhibition, which is interpreted in first instance on the basis of the equal surface activity of both components initiating damage of the mitochondria. The regression coefficients of curves a) and b) are +0.87 and −0.93, respectively. Similar results were obtained with rotenone absent [15]. Interpretation of these results has to consider real effects on the oxidation of succinate, as well as uncoupling effects, brought about by the long chain fatty acid esters. Uncoupling is most easily studied in anaerobic systems with reversed electron transfer responding very sensitively to uncoupling agents.

Fig. 2 shows an experiment with liver mitochondria where the reduction of cytochrome *c* by endogenous substrate is observed during anaerobiosis. Addition of oxaloacetate as a hydrogen acceptor and of ATP results in an oxidation of cytochrome *c* [20]. L(−)PC in concentrations as employed in the other experiments does not cause a release of the energized state, whereas the uncoupler dicoumarol causes an immediate reduction of cytochrome *c*. That means that an acceleration of succinate oxidation by PC can not be due to uncoupling of oxidative phosphorylation, which is also supported by the different response to L(−)- and D(+)PC. Furthermore, an uncoupling effect at concentrations which do not affect the permeability of the mito-

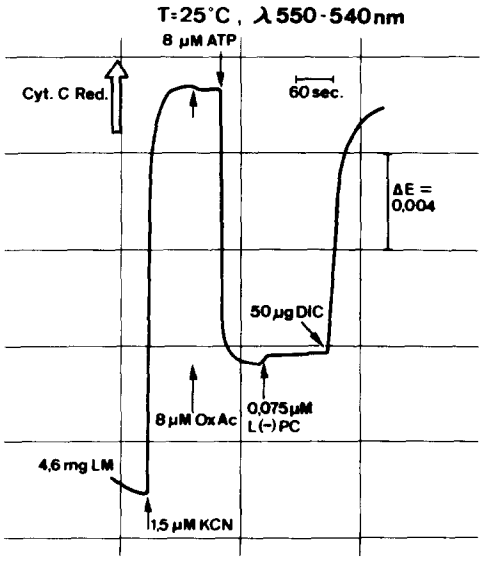

Fig. 2. Separation of the effect of L(−)PC on the respiratory chain from the effects of the uncoupler dicoumarol (DIC). Registration of the absorbancy change of cytochrome *c*; reaction mixture 2.0 ml; all additions indicated in absolute amounts; light path 10 mm. Other details see "methods". Isolations were carried out in the same medium as for isolation of mitochondria.

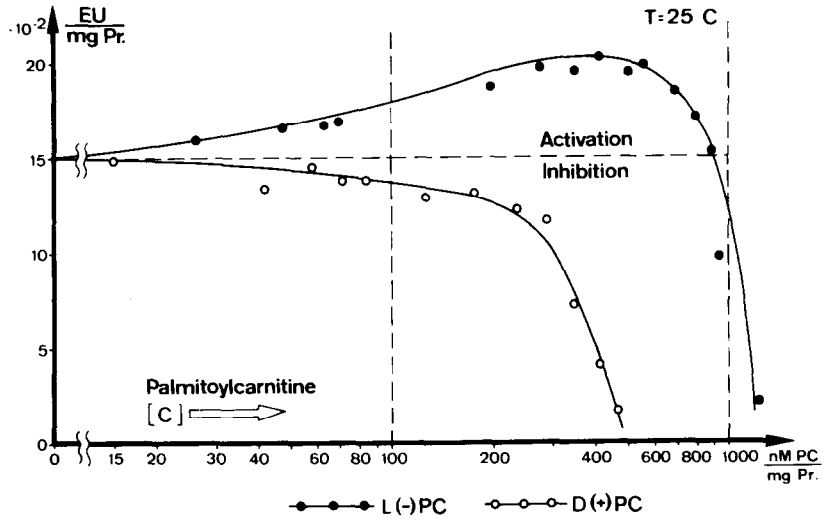

Fig. 3. Effect of increasing concentrations of L(−)PC and D(+)PC on mitochondrial succinate dehydrogenase activity. Measurements with cytochrome *c* (1.33×10^{-4} M) as terminal electron acceptor, 546 nm, lightpath 10 mm; other details described in [16].

chondrial membrane by means of surface activity seems unlikely also with respect to the chemiosmotic coupling theory [21], since the acyl-carnitines are electrically neutral compounds, unable to serve as proton conductors.

In another set of experiments the influence of PC on the activity of mitochondrial succinate dehydrogenase has been studied, as measured with cytochrome *c* as terminal electron acceptor with mitochondria, which have been exposed to hypoosmotic shock and freezing/thawing, in order to break the membranes. Fig. 3 shows that in accordance with the respiratory experiments the oxidation of succinate is enhanced by increasing concentrations of PC and is depressed at high concentrations, where a response to surface activity could no longer be excluded. Interestingly, also in this case the D(+) isomer at lower concentrations was inactive to stimulate succinate oxidation. On the contrary, a slight inhibition of succinodehydrogenase could be observed already at concentrations, where the L(−) compound induced a definite activation. So far the effect reveals to be stereospecific.

In a more detailed kinetic study of the reaction the Lineweaver-Burk plot reveals that in the presence of PC the characteristics of succinate oxidation are changed to a larger V_{max} and to a diminished apparent K_m for succinate (3.3×10^{-5} M rotenone present). From a series of preparations an average K_m of 5.29×10^{-4} M was calculated with PC absent, and of 2.74×10^{-4} M with 2×10^{-5} M L(−)PC present. Lower apparent K_m values of 1.3×10^{-4} M could be observed with L(−)PC concentrations of 3×10^{-4} M.

From this it follows that the normal cellular concentration of succinate [22], which varies between 1.5×10^{-4} and 8×10^{-6} M is below the K_m of SDH. Lowering the apparent K_m of the enzyme by a factor of 2 or of almost 4, as in the presence of higher concentrations of PC, should result in an increased formation of malate from succinate, which in turn causes an enhanced oxaloacetate formation depending on the NADH/NAD^+ ratio. On the other hand, the acceleration of succinate dependent respiration by PC far exceeds that expected from the increased substrate affinity of the enzyme. It appears possible, furthermore, that the accessibility of succinate, which usually is generated inside of the mitochondria, is facilitated by PC.

In addition to exerting control at the SDH step, the energy level of liver mitochondria *in vitro* is decreased by PC, as a consequence of increased acetoacetate formation, when rotenone is absent. This mainly explains the enhancement of the respiratory rate, since acetoacetate serves as an X~I consuming

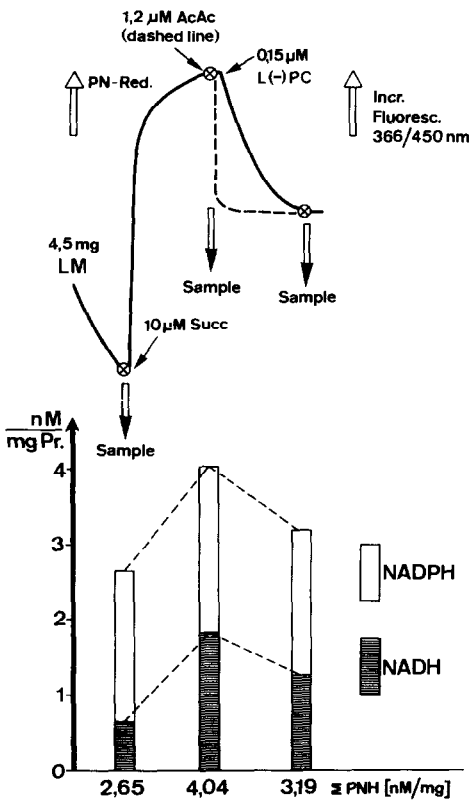

Fig. 4. Oxidation of mitochondrial pyridinenucleotides by L(−)PC. Upper trace: fluorimetric record of mitochondrial PN-reduction (scale expansion arbitrary). Lower part shows results of enzymatic analysis of samples, taken at the indicated points. The dashed trace shows the spontaneous oxidation by acetoacetate addition. Reaction mixture 2.0 ml; all additions indicated in absolute amounts. For other conditions see "methods".

trap for hydrogen flow from succinate to the NADH level [23]. Fig. 4 shows the reduction of mitochondrial pyridinenucleotides (PN) by succinate, which is partially reversed by addition of PC. Samples for enzymatic analysis were taken at times indicated by arrows below the fluorimetric trace. The dashed trace gives the result of an analogous experiment, with addition of a corresponding amount of acetoacetate instead of PC, and it is suggested that the response to PC is slower, presumably resembling the kinetics of acetoacetate formation. The degree of respiratory control is diminished [15], as expected.

Our results are in accordance with those of Williamson et al. [24], reporting a fall of the ATP/ADP ratio during enhanced fatty acid oxidation and ketogenesis. The latter results, however, were obtained with whole liver, which only contains approximately 20% of its ATP intramitochondrially [25]. In any case, on the basis of our and other results [14], it seems questionable, whether ATP is the predominant control factor of citrate formation or ketogenesis resp., since in the presence of PC malate formation is accelerated by modification of the SDH activity, as well as oxaloacetate formation by lowering the energy level and the intramitochondrial concentration of NADH. As demonstrated by Portenhauser [26], during oxidation of palmitate by liver mitochondria, palmitoylcarnitine occurs in the same range of SDH-activating concentrations as employed in the above experiments. Moreover, Norum [27] has reported increased activities of long chain acyl-CoA-carnitine-transferase in rat liver during metabolic situations with increased fatty acid mobilization to the liver. Concomitantly, according to Tubbs [28], in livers of starved, fat fed, and alloxan diabetic rats the levels of long chain acyl-CoA and acyl-carnitines are significantly elevated.

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