Acta Chemica Scandinavica B 30 (1976) 283—286

Regulation and Metabolic Background of Polyketide Formation. I. Effects of (—)-Hydroxycitrate and Metabolic Roles of Citrate and Malate in Fatty Acid and Polyketide Formations

KARL HULT and STEN GATENBECK

Department of Pure and Applied Biochemistry, The Royal Institute of Technology, S-100 44 Stockholm 70, Sweden

The effects of (−)-hydroxycitrate on fatty acid and alternariol syntheses from glucose and acetate in *Alternaria alternata* were investigated.

Fatty acid synthesis from glucose and acetate was inhibited by (−)-hydroxycitrate. The inhibition could partly be removed by the addition of malate or isocitrate. Alternariol synthesis from glucose was also inhibited by (−)-hydroxycitrate. This inhibition was not influenced to the same extent by malate addition as was that of fatty acid synthesis.

It is shown that ATP-citrate lyase is essential not only for the production of cytoplasmic acetyl-CoA but also for the production of NADPH by supplying malate *via* oxaloacetate.

Fatty acid synthesis in animal tissue depends on mitochondrially formed acetyl-CoA which is transported to the cytoplasm as citrate. The cytoplasmic citrate is cleaved by ATP-citrate lyase (EC 4.1.3.8, ATP:citrate oxaloacetate-lyase) into oxaloacetate and acetyl-CoA which is used for fatty acid synthesis.[1]

ATP-citrate lyases from the fungi *Penicillium* [2] and *Mortierella* [3] were recently reported. The fungus *Alternaria alternata* forms in addition to fatty acids also the polyketide alternariol from acetyl-CoA.[4] The biosyntheses of fatty acids and polyketides differ only in the reduction steps which are present in fatty acid synthesis but not in polyketide synthesis.[4] It was therefore decided to test the ATP-citrate lyase system and its influence on the formation of these two groups of compounds of acetyl-CoA origin in *A. alternata*.

(−)-Hydroxycitrate, a powerful inhibitor of ATP-citrate lyase,[5] inhibits fatty acid synthesis in the rat.[6–8] It is assumed that the inhibition depends on a shortage of cytoplasmic acetyl-CoA and Barth *et al.*[8] show that acetate can overcome this inhibition, probably by forming cytoplasmic acetyl-CoA independently of ATP-citrate lyase.

In the present investigation the effects of (−)-hydroxycitrate on fatty acid and polyketide biosyntheses have been studied by incorporation experiments with labelled glucose and acetate.

MATERIALS AND METHODS

Cultural conditions. Alternaria alternata (tenuis) (CMI 89 343) was obtained from the Commonwealth Mycological Institute (Kew, Surrey). Stock cultures of this organism were maintained at 4 °C on agar as described by Jerebzoff.[9] Submerged cultures were incubated from the agar slants in conical flasks of 500 ml capacity containing 150 ml modified Czapek-Dox medium of the following composition: $NaNO_3$, 1.0 g; NH_4Cl, 0.25 g; KH_2PO_4, 1.0 g; $MgSO_4.7H_2O$, 0.5 g; KCl, 0.25 g; NaCl, 0.25 g; $ZnSO_4.7H_2O$, 0.01 g; $FeSO_4.7H_2O$, 0.01 g; yeast extract, 1.0 g; glucose, 40 g and distilled water, 1000 ml. The mycelium was harvested by filtration after 5 to 10 days and washed with modified Czapek-Dox medium containing 5 g glucose per 1000 ml but deficient of nitrogen substrates.

Incubations. Portions of wet mycelium (0.50 g) were incubated in 25 ml conical flasks containing 5 ml modified nitrogen-free Czapek-Dox medium with 5 g glucose per 1000 ml and 2 mM sodium acetate. The flasks were incuba-

ted for 6 h on a table rotary shaker at room temperature. Further additions to the medium were done to the following concentrations and as sodium salts, 4 mM (−)-hydroxycitrate; 2 mM malate; 2 mM isocitrate. The following labelled compounds were used: either 1 μCi [U-^{14}C] glucose and 5 μCi 2-^{14}C-acetate in separate incubations or a combination of 1 μCi [U-^{14}C] glucose and 2.5 μCi 2-^{3}H-acetate in a single incubation. The latter combination was preferred in the later stage of the experiments, saving time and increasing precision.

Incorporation of label. Lipids and alternariol were extracted from the mycelium using Floch's method [10] with chloroform-methanol (2:1, by vol.) with 10 ml solvent for 0.5 g wet mycelium. The filtrate obtained from the extraction was shaken with 2 ml water, and the lower phase washed with 2 ml methanol-water (1:1, by vol.). The organic phase containing lipids and alternariol was evaporated to dryness. The residue was chromatographed on thin layer silica gel in benzene-dioxane-acetic acid (95:25:4, by vol). Lipids (R_F 0.65 − 0.85) and alternariol (R_F 0.36) were localized with a radiochromatogram scanner and observation of fluorescense. The activity was eluted from the gel and counted in a Packard Tri-Carb Scintillation Spectrometer.

Scintillation fluid used was toluene containing 4 g PPO and 50 mg dimethyl-POPOP per 1000 ml. Alternariol was dissolved in 500 μl methanol before adding the scintillation fluid.

Material. 2-^{3}H-Acetate was obtained from the Radiochemical Centre, Amersham, England and [U-^{14}C] glucose and 2-^{14}C-acetate from New England Nuclear, Dreieichenhain, W. Germany. (−)-Hydroxycitrate was prepared from *trans*-aconitic acid by a modification of the method of Martius and Maué.[11] Silica gel plastic sheets were obtained from Schleicher and Schüll, W. Germany.

RESULTS AND DISCUSSION

All results are calculated as percentage of a control incubation without inhibitor or other additions. This procedure allows comparison between different cultures. Means and errors are calculated on the basis of the calculated percentages.

Inhibition with (−)-hydroxycitrate. On addition of (−)-hydroxycitrate to the incubation medium the syntheses of fatty acids and alternariol from glucose are inhibited (Table 1). As (−)-hydroxycitrate inhibits ATP-citrate lyase the inhibitions of the biosyntheses of these compounds demonstrate that this enzyme is essential in *A. alternata* for the production of cytoplasmic acetyl-CoA originating from glucose. With exogenous acetate as precursor fatty acid synthesis is inhibited but not alternariol synthesis (Table 1). The unchanged alternariol synthesis shows that (−)-hydroxycitrate has no influence on the formation of acetyl-CoA and malonyl-CoA from acetate. The inhibition of fatty acid synthesis from acetate must therefore be due to (−)-hydroxycitrate affecting the availability of cytoplasmic NADPH. The main difference between fatty acid and alternariol syntheses, as pointed out earlier, is the demand for NADPH in fatty acid synthesis.

ATP-citrate lyase provides in addition to acetyl-CoA also oxaloacetate. This oxaloacetate is reduced by cytoplasmic NADH to malate with malate dehydrogenase (EC 1.1.1.37, L-malate: NAD^+ oxidoreductase). The cytoplasmic malate is partly utilized in a shuttle system for transport of citrate out of the mitochondria. Inside

Table 1. Effects of (−)-hydroxycitrate, malate, and isocitrate on fatty acid and alternariol syntheses *in vivo* in *Alternaria alternata*. The results are the mean ± S.E.M. of the number of experiments indicated by the figures in parentheses.

Addition	Fatty acid synthesis from glucose %	Fatty acid synthesis from acetate %	Alternariol synthesis from glucose %	Alternariol synthesis from acetate %
Control	100	100	100	100
Hydroxycitrate	40 ± 2 (10)	60 ± 6 (6)	54 ± 4 (9)	107 ± 6 (4)
Hydroxycitrate + malate	68 ± 4 (5)	98 ± 19 (2)	67 ± 2 (4)	118 ± 5 (2)
Hydroxycitrate + isocitrate	66 ± 6 (2)	92 ± 16 (2)	–	112 ± 34 (2)
Malate	93 ± 13 (5)	92 (1)	119 ± 2 (5)	109 (1)

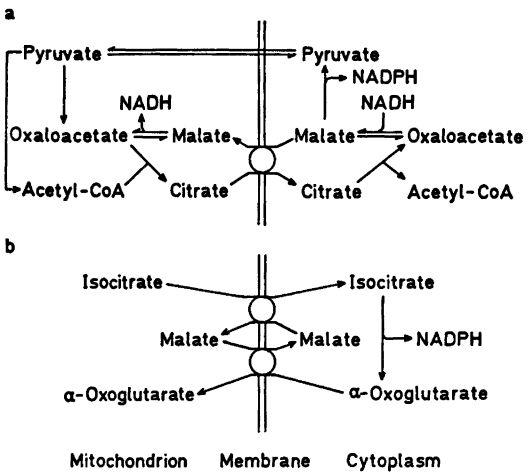

Fig. 1. The roles of malate in cytoplasmic NADPH regeneration processes and mitochondrial shuttle system. a: Formation of cytoplasmic acetyl-CoA from mitochondrial acetyl-CoA. Malic enzyme deprives citrate transport of malate. b: Formation of NADPH by cytoplasmic isocitrate dehydrogenase.

the mitochondria the malate is reoxidized to oxaloacetate and subsequently reutilized for citrate formation (Fig. 1a).

The inhibition caused by (−)-hydroxycitrate can be influenced by the addition of exogenous malate (Table 1). On addition of malate to (−)-hydroxycitrate inhibited cells glucose is incorporated more efficiently into both fatty acids and alternariol. The inhibition of fatty acid synthesis from acetate is entirely removed on addition of malate to (−)-hydroxycitrate inhibited cells. These results are interpreted in the following manner. Since malate alone does not influence fatty acid and alternariol syntheses in the same way as when malate is added to inhibited cells, (−)-hydroxycitrate is likely to induce a shortage of malate. It seems that the addition of malate to inhibited cells mainly influences the production of cytoplasmic NADPH since fatty acid synthesis is the one that is most promoted by malate addition. Besides being involved in the mitochondrial transport of citrate and reducing equivalents, malate thus seems to play a role in the production of NADPH.

Cytoplasmic malic enzyme [EC 1.1.1.40, L-malate:NADP+ oxidoreductase (oxaloacetate-decarboxylating)] will split malate to pyruvate and carbon dioxide with the concomitant formation of NADPH. Malate can also participate in NADPH formation in a more indirect way by being used for transport of isocitrate [12] out of the mitochondria (Fig. 1b). Cytoplasmic isocitrate dehydrogenase [EC 1.1.1.42, *threo*-D-isocitrate:NADP+ oxidoreductase (decarboxylating)] will oxidize isocitrate to α-oxoglutarate and provide NADPH. α-Oxoglutarate is then transported into the mitochondria in exchange for malate.[12] A reduced malate concentration would thus reduce the transport rate of isocitrate and possibly also the production of cytoplasmic NADPH. The participation of isocitrate dehydrogenase *in vivo* is demonstrated by the reduction of the inhibition of the fatty acid synthesis on the addition of isocitrate to (−)-hydroxycitrate inhibited cells (Table 1).

Exogenous isocitrate is able to dilute the radioactivity of citrate formed from labelled glucose in the reaction catalysed by aconitase [EC 4.2.1.3, citrate (isocitrate) hydro-lyase]. This enzyme, however, is inhibited by (−)-hydroxycitrate [13] and such a dilution effect should thus be of no significance.

It is important to take into consideration the role of malate for the transport of citrate, isocitrate and α-ketoglutarate in fatty acid and polyketide syntheses. Every citrate molecule leaving the mitochondria must be exchanged with one malate molecule. If malic enzyme splits malate in the cytoplasm this leads to a loss of malate for transport of citrate and isocitrate. This loss must be met by a cytoplasmic synthesis or by transport of malate from the mitochondria. It is therefore possible that isocitrate dehydrogenase is the principal NADPH producer, depending on but not consuming malate, whereas malic enzyme would mainly have a regulating function by affecting the malate concentration. The inhibition of malate formation induced by (−)-hydroxycitrate thus causes the observed restriction of NADPH formation.

Acknowledgment. This work was supported by grants from the Swedish Natural Science Research Council.

REFERENCES

1. Watson, J. A. and Lowenstein, J. M. *J. Biol. Chem. 245* (1970) 5993.
2. Måhlén, A. *Eur. J. Biochem. 36* (1973) 342.
3. Attwood, M. M. *Antonie van Leeuwenhoek J. Microbiol. Serol. 39* (1973) 539.
4. Gatenbeck, S. and Hermodsson, S. *Acta Chem. Scand. 19* (1965) 65.
5. Watson, J. A., Fang, M. and Lowenstein, J. M. *Arch. Biochem. Biophys. 135* (1969) 209.
6. Lowenstein, J. M. *J. Biol. Chem. 246* (1971) 629.
7. Sullivan, A. C., Triscari, J., Hamilton, J. G., Miller, O. N. and Wheatley, V. R. *Lipids 9* (1974) 121.
8. Barth, C., Hackenschmidt, J., Ullman, H. and Decker, K. *FEBS Lett. 22* (1972) 343.
9. Jerebzoff, S. *C. R. Acad. Sci. Ser. C 254* (1962) 2062.
10. Radin, N. S. *Methods Enzymol. 14* (1969) 245.
11. Martius, C. and Maué, R. *Hoppe-Seyler's Z. Physiol. Chem. 269* (1941) 33.
12. Klingenberg, M. *Essays Biochem. 6* (1970) 119.
13. Cheema-Dhadli, S., Halperin, M. L. and Leznoff, C. C. *Eur. J. Biochem. 38* (1973) 98.

Received September 5, 1975.