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Studies on the Enzyme Synthesizing the Aromatic Product Alternariol

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The properties of an alternariol synthesizing enzyme from *Alternaria tenuis* have been investigated with regard to the influence of time and enzyme concentration on the reaction. Optimum activity was obtained at pH 7.8–7.9 at 28°C. No activation of the enzyme was observed with the metal ions tested but Zn^{2+} and Cu^{2+} inhibited the reaction. Inhibition was also obtained with the usual SH-reagents, poly-β-keto compounds, and coenzyme A as well. On the other hand, the thiol compounds glutathione and cystein stimulated the reaction. Excess of acetyl coenzyme A was found to inhibit the enzyme activity when using malonylpantetheine as cosubstrate. Similarly, increasing amounts of malonyl coenzyme A inhibits the formation of alternariol at constant concentrations of acetyl coenzyme A or acetylpantetheine. K_m for acetyl coenzyme A, acetylpantetheine and malonylpantetheine have been determined by using various combinations of the substrates. Of a number of coenzyme A derivatives tested only propionyl coenzyme A was able to replace acetyl coenzyme A in the condensation with malonyl coenzyme A.

The enzymic synthesis of the acetate-malonate derived aromatic compound alternariol has been recently reported by Gatenbeck and Hermodsson.[1] The enzyme, isolated from *Alternaria tenuis*, was purified 30-fold. Substrates for the aromatic synthesis were acetyl CoA * and malonyl CoA. Attempts at further purification of the enzyme have so far been without success. The difficulties may be, that we probably are dealing with a very labile enzyme complex similar to the fatty acid synthesizing system described by Lynen.[2]

In this publication a more extensive investigation of the properties of the enzyme preparation at the present stage of purification will be presented.

MATERIALS AND METHODS

Malonyl CoA was prepared as described by Lynen.[3] Malonylpantetheine was obtained by the same method after reduction of pantethine in aqueous solution with sodium borohydride. Acetyl CoA and acetylpantetheine were synthesized by using the method

* CoA = coenzyme A.

of Simon and Shemin.[4] Propionyl CoA as well as the CoA derivatives of higher fatty acid homologues, benzoic acid, phenylacetic acid, methylmalonic acid, and malonamic acid were obtained with the method elaborated by Seubert [5] for preparation of capryl CoA. The assay of the enzyme was carried out as described elsewhere [1] with the modifications given in the legends of the figures and the table.

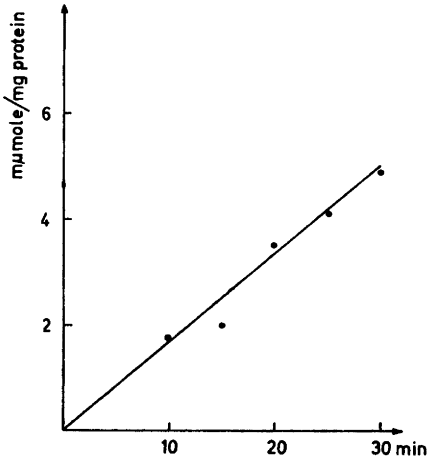

Fig. 1. Enzymic formation of alternariol: dependence of time. Incubation mixture: 2.0 ml (2.1 mg protein) enzyme solution. (Prepared as described by G. and H.[1] with a further addition of glutathione, 10^{-3} M), 1.2 μmoles (0.25 μC) malonyl-2-^{14}C-pantetheine, 0.20 μmole acetyl CoA. Final volume 2.4 ml. Temperature 20°C, pH 7.7.

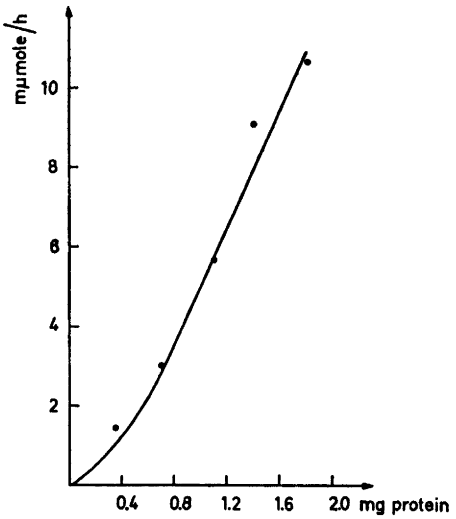

Fig. 2. Enzymic formation of alternariol: dependence on enzyme concentration. Incubation mixture: see Fig. 1, Time 30 min at 20°C, pH 7.7.

RESULTS AND DISCUSSION

With the enzyme concentration used and at 20°C the alternariol formation showed a linear dependence of time over the period investigated. Essentially, a linear dependence was also found for the enzyme activity when plotted against the enzyme concentration. However, at low enzyme concentrations a slight denaturation of the enzyme was observed (Fig. 2). The pH dependence of the reaction was tested over a range from pH 6.8 to 8.2 at 20°C. A distinct optimum was found between 7.8—7.9. The rapid decrease of enzyme activity at a pH above the optimum value is probably caused by hydrolysis of the CoA derivatives and thiolesters eventually formed between the enzyme complex and the added acyl groups. The reaction was also found to proceed at optimal velocity at 28°C (Fig. 4). The enzyme activity was investigated at various levels of substrate concentrations using different combinations of acetyl CoA, acetylpantetheine, malonyl CoA, and malonylpantetheine as substrates (Figs. 5—10). From Fig. 5 which shows the velocity of alternariol

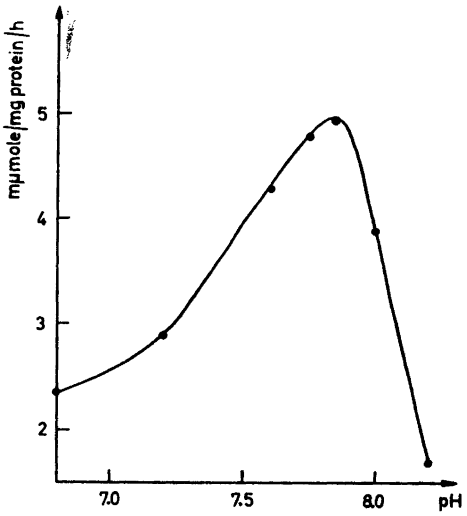

Fig. 3. Enzymic formation of alternariol: dependence of pH. Incubation mixture: see Fig. 1. Time 30 min at 20°C; pH adjusted with 2 M HCl and 1 M NaOH, resp.

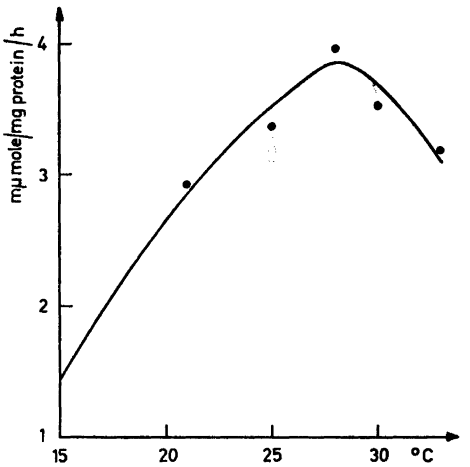

Fig. 4. Enzymic formation of alternariol: dependence of temperature. Incubation mixture: 2.0 ml (2.4 mg protein) enzyme solution, 1.2 μmoles (0.25 μC) malonyl-2-^{14}C-pantetheine, 0.30 μmole acetyl CoA. Final volume 2.4 ml. Time 30 min, pH 7.7.

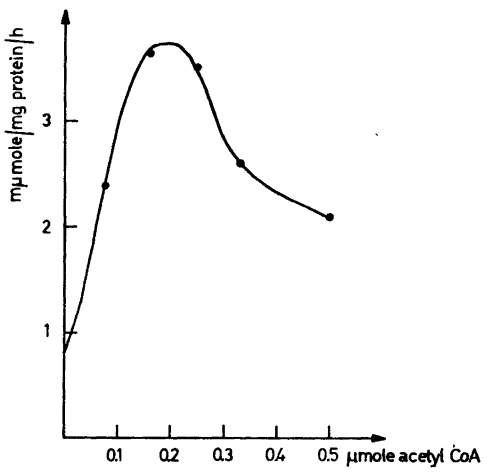

Fig. 5. Enzymic synthesis of alternariol: dependence of acetyl CoA concentration. Incubation mixture: 2.0 ml (2.6 mg protein) enzyme solution, 1.2 μmoles (0.25 μC) malonyl-2-^{14}C-pantetheine, 0—0.50 μmole acetyl CoA. Final volume 2.5 ml. Temperature 20°C, pH 7.7.

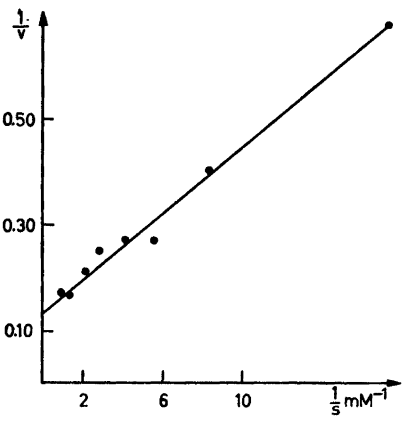

Fig. 6. Determination of K_m for malonyl-pantetheine. Incubation mixture: 1.9 ml (1.45 mg protein) enzyme solution, 0.30—2.40 μmoles malonyl-2-^{14}C-pantetheine, 0.20 μmole acetyl CoA. Final volume 2.5 ml. Time 30 min at 20°C, pH 7.7.

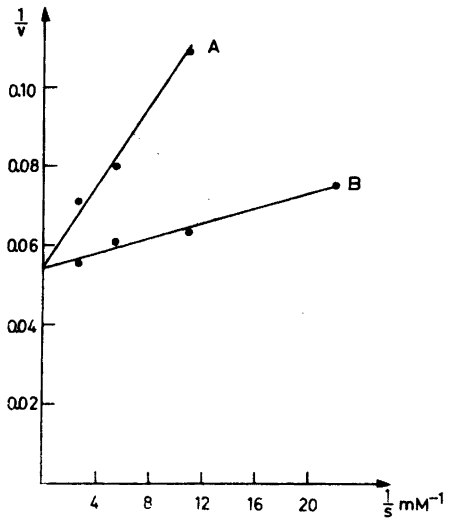

Fig. 7. Determinations of K_m for acetyl CoA and K_i for CoA. Incubation mixture, 1.6 ml (0.8 mg protein) enzyme solution, 1.2 μmoles (0.25 μC) malonyl-2-^{14}C CoA, 0.10—0.80 μmole acetyl CoA, 0.34 mM CoA (curve A). Final volume 2.2 ml. Time 30 min at 20°C, pH 7.7.

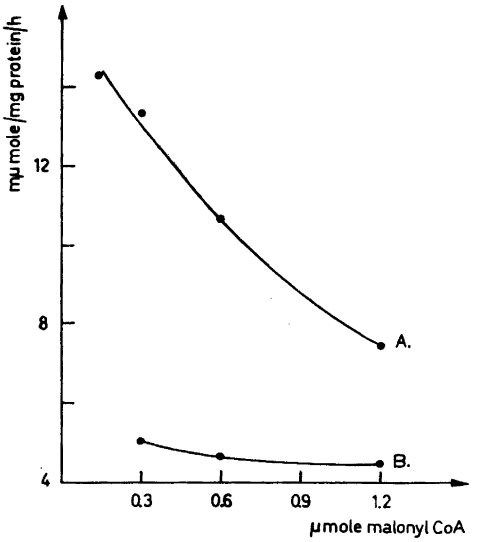

Fig. 8. The influence of malonyl CoA concentration on alternariol synthesis with acetyl CoA as cosubstrate. Incubation mixture: 1.5 ml (0.6 mg protein) enzyme solution, 0.15—1.20 μmoles (0.03—0.25 μC) malonyl-2-^{14}C-CoA, 0.20 μmole acetyl CoA, 0.40 mM CoA (curve B). Final volume 1.9 ml. Time 30 min at 20°C, pH 7.7.

formation in presence of constant amounts of malonylpantetheine and variable amounts of acetyl CoA, it is seen that at a certain level of acetyl CoA the reaction is inhibited. The optimal velocity occurs when the ratio of malonylpantetheine:acetyl CoA is 6:1 which is the ratio of malonate:acetate to be found in the formed alternariol. The inhibition might depend on a certain affinity of acetyl CoA for the malonate binding enzyme. Using a constant level of acetyl CoA and varying the malonylpantetheine concentration the described effect is not obtained but a steadily increasing velocity is observed over the range of malonylpantetheine concentrations used. Apparently malonylpantetheine has a very low, if any, affinity to the acetate binding enzyme. From the kinetic data in Fig. 6, K_m* for malonylpantetheine has been determined to 2.4×10^{-4} M. In Fig. 7 is demonstrated the effects on the enzyme activity of variable amounts of acetyl CoA at constant malonyl CoA concentration. From curve B, Fig. 7, K_m for acetyl CoA was found to be 1.8×10^{-5} M. It was noticed that free CoA acted as an inhibitor of the reaction, and the Lineweaver Burk diagram (Fig. 7) shows that it has the character of a competitive inhibitor (K_i 8.1×10^{-5} M). When keeping acetyl CoA and acetylpantetheine resp. at constant levels increasing amounts of malonyl CoA inhibits the formation of alternariol (Figs. 8 and 9). This effect of malonyl CoA corresponds to that

* The concentrations of the cosubstrates are given in the legend of the figures.

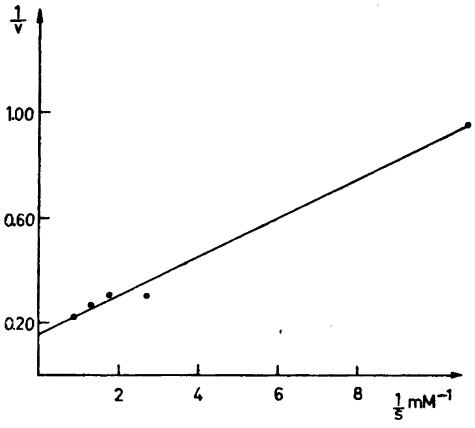

Fig. 9. Determination of K_m for acetyl pantetheine. Incubation mixture: 2.0 ml (1.1 mg protein) enzyme solution, 1.2 μmoles (0.25 μC) malonyl-2-^{14}C-pantetheine, 0.25—3.0 μmoles acetylpantetheine. Final volume 2.7 ml. Time 30 min at 20°C, pH 7.7.

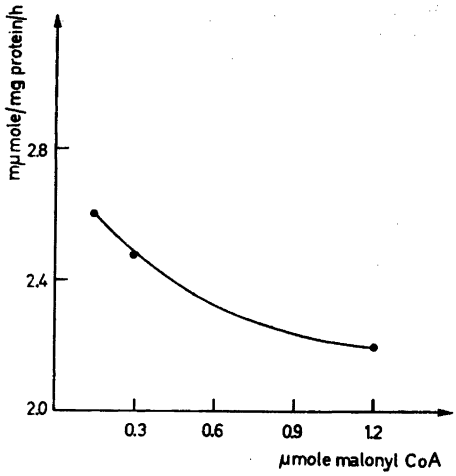

Fig. 10. The influence of malonyl CoA concentration on alternariol synthesis with acetylpantetheine as cosubstrate. Incubation mixture: 1.6 ml (1.0 mg protein) enzyme solution, 0.15—1.20 μmoles (0.03—0.25 μC) malonyl-2-^{14}C CoA, 0.80 μmole acetylpantetheine. Final volume 2.0 ml. Time 30 min at 20°C, pH 7.7.

of acetyl CoA on the malonate binding enzyme and could indicate an affinity of malonyl CoA to the active site of the acetate binding enzyme. K_m for acetylpantetheine (Fig. 10) with malonylpantetheine as cosubstrate was found to be 5.0×10^{-4} M. Comparing the K_m for acetyl CoA (1.8×10^{-5} M) it is evident that acetyl CoA has a considerable higher affinity to the enzyme. Furthermore, Fig. 10 does not indicate any affinity of acetylpantetheine to the malonate binding enzyme.

The influence on the enzyme activity of metal ions, SH-compounds and some other substances has been investigated (Table 1). None of the metal ions used showed any activating effect but the presence of Zn^{2+} and Cu^{2+} had a pronounced inhibitory influence on the reaction. Addition of EDTA did not cause any change of the enzyme activity indicating no requirement of metal ions as cofactors. The SH-compounds glutathione and cystein activated the enzyme. This behaviour towards SH-compounds, activation by glutathione and cystein but inhibition by CoA has this enzyme in common with the fatty acid synthesizing complex which further emphasizes the similarity between the two systems. Another property in common for the two systems is the inhibitory effect of palmitoyl CoA.

The usual SH-reagents shown in Table 1 exhibit a strong inhibitory effect on the enzyme activity indicating the presence of SH groups of importance in the enzyme. The inhibition of, *e.g.*, *p*-chloromercuribenzoate is prevented by preincubation with glutathione.

Table 1. The effects of metal ions, SH-compounds, SH-reagents *etc.* on the enzyme activity. Incubation mixture: 2.0 ml (1.4 mg protein) enzyme solution (minus glutathione and EDTA), 1.2 μmole (0.25 μC) malonyl-2-^{14}C-pantetheine, 0.20 μmole acetyl CoA. Final volume 2.4 ml. Time 30 min at 25°C, pH 7.7.

Substance added	Concentration of added substance (M)	% Inhibition
Mg^{2+}	10^{-3}	0
Mn^{2+}	10^{-3}	25
Zn^{2+}	10^{-3}	87
Cu^{2+}	10^{-3}	89
Fe^{2+}	10^{-3}	0
Glutathione	10^{-4}	+ 13[a]
»	10^{-3}	+ 40[a]
Cystein	10^{-4}	+ 8[a]
»	10^{-3}	+ 15[a]
p-Chloromercuribenzoate	10^{-4}	72
»	10^{-5}	42
»	10^{-6}	35
Phenylmercuriacetate	10^{-4}	80
»	10^{-5}	64
»	10^{-6}	26
Iodoacetamide	10^{-3}	100
»	10^{-4}	44
Sodium citrate	10^{-2}	0
Sodium isocitrate	10^{-2}	0
Sodium malate	10^{-3}	0
Sodium fumarate	10^{-3}	0
Palmitoyl CoA	10^{-3}	30
Acetylacetone	10^{-3}	19
Ethyl acetoacetate	10^{-3}	11
Acetonylacetone	10^{-3}	6

[a] The reaction is activated.

Addition of citric acid cycle intermediates such as citrate, isocitrate, malate, and fumarate to the reaction mixture had no influence on the reaction velocity.

A possible mechanism for the formation of alternariol and other aromatic compounds of acetate-malonate origin as well, is that the acetate binding enzyme and the malonate binding enzyme(s) form a multienzyme package in which the initial condensation is a reaction between the protein bound acetate and a protein bound malonate unit in an appropriate position giving rise to a protein bound β-keto C_4 unit (*cf.* fatty acid synthesis). The further extensions of the β-keto C_4 unit include poly-β-keto intermediates bound to the enzyme surface possibly by hydrogen bonds. The release of the product from the enzyme surface occurs in the moment aromatization takes place.

If the proposed mechanism is valid it would be possible to compete for the positions of the hydrogen bonds by adding a poly-β-keto compound to the

incubation mixture and consequently inhibiting the reaction. In Table 1 a few polyketo substances are listed. Among these compounds acetylacetone and ethylacetoacetate exhibit stronger inhibition than the β,ε-diketo compound acetonylacetone in agreement with the hypothesis.

SPECIFICITY OF THE ENZYME

A vast number of compounds isolated from microorganisms and plants have been shown to be derived from acetate-malonate. Biogenetically related to this group, the acetogenins, are a few other groups of substances the formation of which can be visualized by replacing the acetate unit in the acetogenins with another acyl groups. Some examples are flavonoids, pyrromycinons, and tetracyclines in which acetate has been replaced with cinnamate, propionate, and malonamate, respectively.

In order to investigate the possibility of synthesizing other diphenyl compounds than alternariol, acetyl CoA was substituted in the incubation mixture with propionyl CoA, butyryl CoA, isobutyryl CoA, benzoyl CoA, phenylacetyl CoA, methylmalonyl CoA, and malonamyl CoA, respectively. Of these CoA derivatives only propionyl CoA yielded together with malonyl CoA a condensation product with the expected paper chromatographic properties. This condensation product could be obtained ^{14}C-labelled either from malonyl-^{14}C-pantetheine or propionyl-^{14}C CoA with a velocity of 20 % of that of alternariol synthesis.

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