THE STEREOSPECIFICITY OF THE (R)-CITRATE SYNTHASE IN THE PRESENCE OF p-CHLOROMERCURIBENZOATE

S. Dittbrenner, A. A. Chowdhury and G. Gottschalk

Institut für Mikrobiologie der Universität
34 Göttingen, Goßlerstraße 16, Germany

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Summary: The stereospecificity of partially purified (R)-citrate synthase from Clostridium acidi-urici and from C. kluyveri was determined in the presence of p-chloromercuribenzoate. A change of the stereospecificity from the (R)-type to the (S)-type could not be observed.

In 1966 it was reported that Clostridium kluyveri contains an atypical citrate synthase (1) and that the presence of this type of enzyme in C. kluyveri accounts for the unusual origin of the carbon atoms of glutamate first observed by Tomlinson in 1954 (2). This enzyme, now called (R)-citrate synthase, has been found only in a small group of anaerobes: in C. acidi-urici, C. cylindrosporum, Desulfovibrio desulfuricans, and D. vulgaris (3, 4). The majority of anaerobic bacteria that are able to synthesize glutamate via the reactions of the tricarboxylic acid cycle contains a citrate synthase with the usual (S-type) stereospecificity. Among these is the so-called Worthington strain of C. kluyveri (5).

Recently, it was suggested by O'Brien and Stern (6) that the stereospecificity of the (R)-citrate synthase of C. kluyveri could be

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reversibly changed to the (S)-type by incubating the cell-free extract with p-chloromercuribenzoate (pCMB). Furthermore, it was assumed that this change of the stereospecificity of the enzyme accounted for a number of contradictory results on the stereochemistry of citrate synthesis in C. kluyveri.

In this paper we report the effect of pCMB on the stereospecificity of partially purified (R)-citrate synthase from C. acidi-urici and C. kluyveri.

METHODS

C. acidi-urici and C. kluyveri were grown using the media of Rabinowitz (7) and of Stadtman and Barker (8), respectively. Cell-free extracts were prepared from frozen cells as previously described (1).

(R)-citrate synthase was purified from C. acidi-urici according to the procedure recently published (9). Ammonium sulfate was added to the DEAE-Sephadex eluate to give a 70 % saturation. After centrifugation the precipitate was dissolved in 4 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 100 mM KCl and 0.1 mM EDTA. The enzyme solution was dialyzed for 8 hours against 1 000 ml of the same buffer; the buffer was changed once. The freshly prepared enzyme had a specific activity of 0.8 U/mg protein and was purified about 150-fold as compared to the activity of the crude extract.

A 10-fold purified (R)-citrate synthase preparation (specific activity: 6.5 U/g of protein) from C. kluyveri was obtained by carrying out the first two steps of the procedure employed for the C. acidi-urici enzyme. The protein solution was concentrated and dialyzed as described above.

RESULTS

The effect of pCMB on the activity of the (R)-citrate synthase from C. acidi-urici and from C. kluyveri is shown in Table 1. At a concentration of $4 \cdot 10^{-5}$ M pCMB the enzyme from C. acidi-urici is inhibited to the extent of 73.4 % and the enzyme from C. kluyveri

Table 1

Inhibition of (R)-Citrate Synthase Activity by pCMB

pCMB (μM)	(R)-Citrate Synthase from C. acidi-urici		(R)-Citrate Synthase from C. kluyveri	
	Citrate Formed (μmoles/10 min)	Inhibit. (%)	Citrate Formed (μmoles/10 min)	Inhibit. (%)
0	0.90	0	0.060	0
2	0.83	7.4	-	-
10	0.53	41.2	0.066	0
20	0.32	64.5	0.054	10
40	0.24	73.4	0.044	26.7
100	0.11	87.8	0.018	70.0
200	-	-	0.014	76.7

The reaction mixture contained in a final volume of 2.0 ml: K-phosphate buffer, pH 8.0, 50 mM; $MnCl_2$, 0.5 mM; K-oxaloacetate, 5 mM; KLi-1-^{14}C-acetyl phosphate (23 000 cpm/μmole), 2.5 mM; coenzyme A, 1 mM; phosphotransacetylase, 10 μg; pCMB as indicated and (R)-citrate synthase preparation, 0.2 mg (C. acidi-urici) and 1.8 mg (C. kluyveri). Protein solutions, buffer, $MnCl_2$ and pCMB were preincubated for 10 min at 30°C, the remaining components of the reaction mixture were then added and the reaction was run for 10 min. The amount of radioactive citrate formed was determined as described previously (9).

to 26.7 % compared to the initial activity. Since the specific activity of the synthase from C. kluyveri was only about 1 % of the activity of the C. acidi-urici enzyme, the protein concentrations in the assay mixtures differed considerably (0.2 and 1.8 mg of protein, respectively). O'Brien and Stern (6), using 26 to 33 mg of protein per assay observed

Table 2

Effect of Oxidizing Conditions and of pCMB on the Stereospecificity of the (R)-Citrate Synthase

Source of Citrate Synthase	Conditions	Radioactivity in Acetate (cpm)	(%)	Malate (cpm)	(%)
C. acidi-urici	anaerobic	240 000	99.4	1 300	0.6
" " "	aerobic	114 100	98.8	1 300	1.2
" " "	" + pCMB	68 000	97.7	1 600	2.3
C. kluyveri	anaerobic	39 000	97.1	1 150	2.9
" "	aerobic	59 800	95.3	2 960	4.7
" "	" + pCMB	15 100	88.9	1 890	11.1

The reaction mixture contained in a final volume of 2.0 ml: K-phosphate buffer, pH 7.8, 50 mM; $MnCl_2$, 0.5 mM; K-α-ketoglutarate, 2 mM; KLi-acetyl phosphate, 2.5 mM; coenzyme A, 1 mM; DL-4-^{14}C-aspartate (2.5 μC/μmole), 1 mM; glutamate-oxaloacetate transaminase, 20 μg; phosphotransacetylase, 10 μg; and (R)-citrate synthase preparation as in Table 1. Anaerobic conditions were attained by incubating the reaction mixtures in the presence of 20 mM 2-mercaptoethanol under nitrogen gas. In Exp. 3 and 6 the pCMB concentration was 0.1 and 0.2 mM, respectively. After preincubation for 20 min the reactions were allowed to proceed for 1 hour. Radioactive citrate was isolated from the mixtures by column and paper chromatography. Aliquotes of the citrate samples were cleaved by citrate lyase in the presence of malate dehydrogenase and the resulting products, acetate and malate, were separated on Dowex-1-formate. This procedure has been recently described in detail (3).

a 35 % inhibition of citrate synthase activity by $1 \cdot 10^{-3}$M pCMB in crude cell-free extracts of C. kluyveri.

The stereospecificity of the partially purified (R)-citrate synthase preparations was determined under three different conditions: under nitrogen in the presence of 2-mercaptoethanol, under air in the absence of 2-mercaptoethanol and in the presence of a pCMB concentration which caused an inhibition of the enzyme activity of 87.8 % (C. acidi-urici) and 76.7 % (C. kluyveri), respectively. The results are summarized in Table 2. The percentage of radioactivity present in acetate gives an indication of the quantitative contribution of the (R)-citrate synthase to citrate synthesis; the radioactivity in malate, on the other hand, reflects the contribution of the (S)-citrate synthase. It can be seen that there was a small increase of radioactivity in malate when the reactions were carried out in the presence of pCMB. This increase may be explained by assuming that a pCMB-insensitive (S)-synthase was present in small concentrations. The contribution of this enzyme to citrate synthesis would then be relatively greater under conditions which cause a pronounced inhibition of the (R)-synthase.

DISCUSSION

These experiments do not lead to the conclusion that the (R)-citrate synthase can be converted into a (S)-synthase by treatment with pCMB. The relative increase of (S)-synthase activity under conditions under which the (R)-synthase is largely inhibited is apparently effected by the presence of a small amount of pCMB-insensitive (S)-

synthase in the enzyme preparations. It is known that the (S)-citrate synthase of some sources is not inhibited by thiol reagents (10, 11); the enzyme from E. coli is inhibited only in solutions of low ionic strength (12).

O'Brien and Stern (6) suggested that a possible dependence of the stereospecificity of the citrate synthase on the redox state might explain contradictory results on the origin of the glutamate carbon atoms of C. kluyveri. This seems to be unlikely since C. kluyveri produces large amounts of hydrogen gas during growth and the environment should, therefore, always be reduced. Possible explanations for the discrepancies are: C. kluyveri contains the genetic information for the synthesis of both synthases; strains of C. kluyveri may contain either (R)- or (S)-citrate synthase; some cultures used may have been contaminated with microorganisms containing (S)-synthase.

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