REVERSAL OF THE STEREOSPECIFICITY OF THE CITRATE SYNTHASE OF *CLOSTRIDIUM KLUYVERI* BY p-CHLOROMERCURIBENZOATE*

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Summary: The stereospecificity of citrate synthase in extracts of *Clostridium kluyveri* (Barker strain) can be changed reversibly from the (R)-type to the (S)-type by treatment with p-chloromercuribenzoate. It is proposed that sulfhydryl groups are essential for the (R)-type stereospecificity of the enzyme and their incomplete oxidation or reduction accounts for mixed labelling patterns in citrate.

In *Clostridium kluyveri* glutamate is synthesized via citrate synthase and other enzymes of the citric acid cycle (1,2,3). Studies of ^{14}C transfer from precursors into carbons 1 and 5 of glutamate led to different conclusions about the stereospecificity of the citrate synthase.

Gottschalk and Barker (2,4) found that the citrate synthase in extracts of the Barker strain had an opposite stereospecificity (*i.e.* formed (R)-citrate) to that shown by the pig heart enzyme which formed (S)-citrate; whereas Stern *et al.* (5) found that extracts of cells grown commercially (Worthington) exhibited the (S)-type stereospecificity as did growing cells (6). Extracts of the Leicester strain of *C. kluyveri* (I. Kennedy and J. G. Morris, personal communication) also formed (S)-citrate. The situation was further complicated by the finding of a mixed labelling pattern in

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glutamate synthesized by extracts of C. kluyveri indicating intermediate formation of (R)- and (S)-citrates(3).

This paper shows that the (R)-type stereospecificity of citrate synthesis in extracts of the Barker strain can be changed reversibly to the (S)-type by oxidizing conditions and by treatment with p-chloromercuribenzoate (pCMB).

MATERIALS AND METHODS

Growth and Extraction of Cells. Dried cells of C. kluyveri, Barker strain (kindly provided by Dr. H. A. Barker), were prepared according to Stadtman and Burton (7). Extracts were made by autolysis of dried cells in 0.05 M potassium phosphate buffer pH 7.5 at 5°. Washed frozen cells of C. kluyveri (derived from an original Barker strain) were obtained from Worthington Biochemical Corp. Cell extracts were prepared by sonication in the same buffer.

Synthesis and Degradation of Citrate-^{14}C. Citrate-^{14}C was synthesized from oxalacetate-4-^{14}C and acetyl-CoA (Table 1). Cell extracts were incubated 5 min at 26° with pCMB. Unreacted pCMB was removed by adding enough CoASH to react with added pCMB and leave a final concentration of 1 mM. Mercaptoethanol, 20 mM, was also added in Expt. 6, and the reaction tube was flushed with and sealed under hydrogen. In experiments 3 to 6, the substrates were added after 10 min, followed by the coupling enzymes. After 60 more min at 30°, the reaction was stopped by heating at 100° for 3 min. The citrate-^{14}C was isolated and the radioactivity of C-5 determined enzymatically (8). Total radioactivity in C-1 plus C-5 was determined according to Weinhouse et al.(9).

RESULTS

The distribution of ^{14}C in citrate synthesized from oxalacetate-4-^{14}C and acetyl-CoA by extracts of the Barker and Worthington cells are shown in Table 1. Under reducing conditions, with extracts prepared under hydrogen in buffer containing 2-mercaptoethanol and the reaction run under hydrogen, 6% of the total radioactivity (C-1 plus C-5) was found in the C-5 position. With pig heart enzyme almost 100% of the ^{14}C of oxalacetate-4-^{14}C would be found in C-5 of (S)-citrate. Thus the (R)-type stereospecificity predominated as previously reported (2,4). When the extract was prepared and incubated in air, in absence of mercaptoethanol, the ^{14}C content in C-5 rose to 24% (Expt. 2). Thus oxidizing conditions increased the amount of (S)-citrate formed from 6 to 24%.

This result suggested that citrate synthase possessed readily oxidizable groups, e.g., sulfhydryl groups. Therefore, the effect of the alkylating agent pCMB on the labelling pattern of citrate was examined. Pre-treatment of the cell extract with increasing concentrations of pCMB (Expts. 3 to 5) caused the proportion of radioactivity in C-5 of citrate to increase until, with the highest concentration of pCMB (5 mM), 100% of the radioactivity was found in this position. Thus the stereospecificity of the synthase had changed completely from the unusual (R)-type to the usual (S)-type. This change caused by pCMB could be reversed. When mercaptoethanol was added to the extract after treatment with 1 mM pCMB and the incubation carried out under hydrogen (Expt. 6), only 20% of the radioactivity was present in C-5 compared to 75% when the mercaptoethanol and hydrogen were omitted (Expt. 4).

Table 1
Distribution of ^{14}C in Citrate Formed from Oxalacetate-4-^{14}C

Cells	pCMB conc.	Citrate degraded	C1 + C5 Carboxyls $^{14}CO_2$ formed		C5 Carboxyl $^{14}CO_2$ formed	
	mM	dpm	dpm	%	dpm	%
Barker	0	14632	13644	93	787	6
	0	14697	14103	96	3408	24
	0.05	9006	8437	94	4815	57
	1.0	19762	19347	98	14478	75
	5.0	13900	13082	94	13214	101
	1.0	4833	4673	97	914	20
Worthington	0	17506	17395	99	16817	97
	5.0	20416	19213	94	19130	100

The reaction contained in 1.7 ml: potassium phosphate buffer (pH 7.5), 100 μmoles; CoA, 1.5 μmoles; acetyl phosphate, 50 μmoles; L-aspartic acid-4-^{14}C (2.5 μc), 5 μmoles; α-ketoglutarate, 50 μmoles; glutamate-oxalacetate transaminase, 200 μg; phosphotransacetylase, 10 μg and cell extract (33-40 mg. protein). Incubated 1 hr at 30°. For Expt. 1 the extract was prepared in buffer containing 25 mM 2-mercaptoethanol under hydrogen gas. All other experiments used extracts prepared under air. Expts. 1 and 6 were performed under hydrogen; all others in air.

The effect of pCMB on citrate synthesis in extracts of Worthington cells was investigated since they show the (S)-type stereospecificity under reducing conditions (5). As shown in Table 1 when the incubation was carried out under air, 97% of the radioactivity was found in C-5 (Expt. 7) and this was unchanged by treatment with 5 mM pCMB (Expt. 8). Thus the stereospecificity of the Worthington cells, unlike that of Barker cells, was unaffected by oxidizing conditions or by pCMB treatment. Moreover, a variety of reducing treatments applied to the Worthington extract failed to shif its (S)-type stereospecificity toward the (R)-type.

The effect of pCMB concentration on the rate of enzymatic citrate synthesis is shown in Table 2. At 0.05 mM, pCMB caused little inhibition of citrate synthesis in extracts of the Barker strain;

Table 2
Effect of pCMB on Activity of Citrate Synthase

pCMB conc.	Barker Cells Citrate	Barker Cells Inhibition	Worthington Citrate
mM	μmole/mg Pr/hr	%	
0	0.043	0	0.057
0.05	0.040	8	0.054
1.0	0.028	35	0.067
5.0	0.025	42	0.065

The reaction contained in 1.6 ml: potassium phosphate buffer (pH 7.5), 100 μmoles; CoA, 1.5 μmoles; acetyl phosphate, 20 μmoles; potassium oxalacetate, 20 μmoles; phosphotransacetylase, 10 μg and cell extract (26-33 mg protein. Incubated 1 hr at 30°. All extracts prepared and incubated under air; reaction stopped by adding 0.2 ml of 30% trichloroacetic acid and citrate determined according to Stern (10).

1 mM and 5 mM resulted in 35 and 42% inhibition. On the other hand, the rate of citrate synthesis by extracts of Worthington cells was not inhibited by pCMB.

DISCUSSION

These experiments confirm that the Barker strain exhibits essentially the (R)-type stereospecificity of citrate synthesis, whereas the Worthington cells possess essentially the (S)-type stereospecificity under reducing conditions. The stereospecificity of citrate synthesis in the Barker strain could be shifted toward the (S)-type partially by oxidizing conditions and completely by treatment with pCMB. This shift in the labelling patter caused by pCMB could be largely reversed by reducing conditions. However, the labelling pattern of citrate in the Worthington extract was unaffected by oxidizing conditions or by pCMB treatment indicating that its citrate synthase was probably in the "oxidized"form.

It therefore appears that exposed sulfhydryl groups (i.e.,

available to PCMB) are essential for the enzyme to exhibit the (R)-type stereospecificity and that titration of these groups with pCMB converts the enzyme to an oxidized or alkylated form that exhibits the (S)-type stereospecificity. It has not been possible to effect any change of the (S)-type stereospecificity of the Worthington or pig heart enzymes by a variety of reducing conditions (O'Brien and Stern, unpublished experiments), indicating that they must differ from the enzyme in the Barker strain.

The sulfhydryl hypothesis can explain the reported differences in the proportions of (R)- to (S)-citrate formation (3,4,5) in terms of incomplete oxidation or reduction of citrate synthase.

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