SHORT COMMUNICATIONS

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Reversal of the stereospecificity of the citrate synthase of *Clostridium acidi-urici* and *Clostridium cylindrosporum*

Extracts of several anaerobic bacteria[1–3] have been shown to synthesize (*R*)-citrate as measured by the distribution of ^{14}C in citrate synthesized from labeled oxaloacetate or acetyl-CoA. Thus, their citrate synthase had an opposite stereospecificity to that shown by pig heart synthase (citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7) which forms (*S*)-citrate exclusively[4]. O'BRIEN AND STERN[5] have shown that the (*R*)-type stereospecificity of the synthase present in extracts of *Clostridium kluyveri* (Barker) was exhibited only under reducing conditions and that oxidizing conditions caused a significant increase in the amount of (*S*)-citrate formed. The stereospecificity of the *C. kluyveri* synthase could be changed in a reversible manner from the (*R*)-type to the (*S*)-type by treating the extract with *p*-chloromercuribenzoate (PCMB). These observations suggested that exposed sulfhydryl groups (*i.e.* available to PCMB and to O_2) were essential for the enzyme to exhibit the (*R*)-type stereospecificity. We have examined the citrate synthases of *Clostridium acidi-urici* and of *Clostridium cylindrosporum* both reported to form (*R*)-citrate[2] and find that their stereospecificity can be reversed by O_2 or by PCMB.

Frozen and lyophilized cells of *C. acidi-urici* and *C. cylindrosporum* HCl were provided by Dr. J. C. Rabinowitz. Frozen cells of *C. acidi-urici* were dried *in vacuo* over P_2O_5 at 5°. The preparation of cell extracts by sonication (1 min), treatment with PCMB and preincubation with thiols were all carried out as previously described[5]. [^{14}C]Citrate was synthesized from [4-^{14}C]oxaloacetate and acetyl-CoA. After isolation of [^{14}C]citrate, the total ^{14}C in C-1 *plus* C-5 was determined by acid degradation[5]. The radioactivity in C-1*, which was determined as $^{14}CO_2$ using citritase and oxaloacetate decarboxylase[5], corresponded to (*S*)-citrate formation. (*R*)-Citrate formation, represented by ^{14}C in C-5, was calculated by difference.

The proportions of (*R*)- and (*S*)-citrates synthesized from [4-^{14}C]oxaloacetate under various conditions are shown in Table I. When an extract of frozen cells of *C. acidi-urici* was incubated under reducing conditions with 20 mM mercaptoethanol under H_2, 69% of [^{14}C]citrate was in the (*R*)-form and 31% in the (*S*)-form. Replacing 2-mercaptoethanol with dithiothreitol did not alter this pattern. Thus the (*R*)-type stereospecificity, while predominant, was not exclusive as reported by GOTTSCHALK AND BARKER[2] under reducing conditions. When the extract was incubated in air, the amount of (*S*)-citrate formed increased to 55% of the total (Expt. 3). Treatment with PCMB increased (*S*)-citrate formation to 91% of the total, *i.e.* the stereospecificity had been changed almost completely to the (*S*)-type. Under reducing conditions an extract of dried cells of *C. acidi-urici* formed the same proportion of (*R*)- and (*S*)-

Abbreviation: PCMB, *p*-chloromercuribenzoate.

* In accord with a new proposal that the *pro-R* position receive the lower number, the numbering of citrate carbons has been changed and is the reverse of that used in previous papers from this laboratory.

TABLE I

CITRATE ENANTIOMERS FORMED FROM [4-^{14}C]OXALOACETATE AND ACETYL-CoA

The reaction contained in 1.7 ml: 100 μmoles potassium phosphate buffer (pH 7.4); 1.5 μmoles CoA; 50 μmoles acetyl phosphate; 5 μmoles L-[4-^{14}C]aspartic acid (2.5 μC); 50 μmoles α-ketoglutarate; 200 μg glutamate–oxaloacetate transaminase, 10 μg phosphotransacetylase; and cell extract (13–20 mg protein). Incubation time, 1 h at 30°. In Expts. 1, 2, 5, 7 and 10 extract was preincubated with thiol indicated for 1 h at 0° under H_2. These experiments were performed under H_2, the others under air. Citrate synthase activity was assayed as described by O'BRIEN AND STERN[5].

Cells	*Expt. No.*	*Treatment*		*Total radioactivity in C-1 + C-5 (%)*	*Citrate*		*Synthase activity (μmoles/h per mg)*
		2-Mercaptoethanol (mM)	*PCMB (mM)*		*(R) (%)*	*(S) (%)*	
C. acidi-urici							
Frozen	1	20	—	97.0	69.2	30.8	0.062
	2	20*	—	92.7	70.0	30.0	—
	3	—	—	97.7	45.5	54.5	0.279
	4	—	5	90.7	9.4	90.6	0.104
Dried	5	20	—	97.3	63.3	36.7	0.079
	6	—	—	92.2	28.4	71.6	0.075
Lyophilized	7	20	—	99.0	21.3	78.7	—
	8	—	—	94.3	0.2	99.8	0.122
	9	—	5	88.3	1.9	98.1	0.048
C. cylindrosporum							
Lyophilized	10	20	—	92.9	67.7	32.3	0.051
	11	—	—	93.1	5.1	94.9	0.023
	12	—	5	—	—	—	0.021

* Dithiothreitol replaced 2-mercaptoethanol.

citrates as the frozen cell extract (Expt. 5); under oxidizing conditions, it showed a more pronounced shift toward (*S*)-citrate formation (Expt. 6) than did the latter.

An extract of lyophilized *C. acidi-urici* cells incubated under reducing conditions synthesized only 21% of (*R*)-citrate (Expt. 7) and showed a reversal of the ratio of (*R*)- to (*S*)-enantiomers formed when compared to frozen or dried cell extracts similarly treated. When incubated under oxidizing conditions (Expt. 8), it manifested an essentially complete (*S*)-type stereospecificity so that treatment with PCMB was without further effect (Expt. 9). It was evident that lyophilization had resulted in considerable oxidation of the citrate synthase.

Extracts of lyophilized cells of *C. cylindrosporum* tested under reducing conditions (Expt. 10) gave essentially the same proportion of (*R*)- and (*S*)-citrates as did extracts of frozen or dried cells of *C. acidi-urici* but oxidizing conditions alone were sufficient to produce an almost completely (*S*)-type stereospecificity of the synthase (Expt. 11). GOTTSCHALK AND BARKER[2] found that 3–20% of the [^{14}C]citrate formed under reducing conditions by extracts of frozen cells of *C. cylindrosporum* was (*S*)-citrate.

The specific activity of citrate synthase in each type of extract was also determined (Table I). Extracts of *C. acidi-urici* generally had a higher synthase activity

in air than in hydrogen, while the reverse was true of *C. cylindrosporum*. PCMB inhibited the *C. acidi-urici* synthase 61–63% but did not significantly inhibit the *C. cylindrosporum* synthase. There was no correlation between the specific activity of the synthase and the ratio of (*R*)- to (*S*)-citrates formed. Thus a large shift in this ratio occurred without a significant change in the specific activity of synthase (Expt. 5 *versus* Expt. 6). This finding strongly supports the presence of a single synthase rather than of two separate enzymes of opposite stereospecificity.

These experiments demonstrated that under reducing conditions a mixture of (*R*)- and (*S*)-citrates was formed by each type of cell extract, although formation of (*R*)-citrate generally predominated and that oxidizing conditions resulted in an increased, or even exclusive, formation of (*S*)-citrate at the expense of (*R*)-citrate. Since these extracts had been prepared under oxidizing conditions (sonication in air) this change in stereospecificity of the citrate synthase was at least partly reversible. In air, PCMB treatment changed the enzyme product from a mixture of (*R*)- and (*S*)-enantiomers to practically all (*S*)-citrate. Thus, as previously shown for the *C. kluyveri* synthase[5], exposed sulfhydryl groups appear to be essential for the citrate synthase of *C. acidi-urici* and of *C. cylindrosporum* to exhibit the (*R*)-type of stereospecificity. Incomplete oxidation, alkylation or reduction of these groups results in formation of mixtures of (*R*)- and (*S*)-citrates, while after their complete oxidation or alkylation the enzyme displays the (*S*)-type stereospecificity. A significant finding was that drying or lyophilization of whole cells partly converted their citrate synthase to the "oxidized" form.

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1 G. GOTTSCHALK AND H. A. BARKER, *Biochemistry*, 5 (1966) 1125.
2 G. GOTTSCHALK AND H. A. BARKER, *Biochemistry*, 6 (1967) 1027.
3 G. GOTTSCHALK, *European J. Biochem.*, 5 (1968) 346.
4 J. R. STERN, *Abstr. Am. Chem. Soc. New York*, (1966) No. C48.
5 R. W. O'BRIEN AND J. R. STERN, *Biochem. Biophys. Res. Commun.*, 34 (1969) 271.

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