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Enzyme-Catalyzed Synthesis of Citric Acid Using Acetyl-Coenzyme A Recycling in a Two-Phase System

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This paper reports the first demonstration of a two-phase aqueous–organic system combining enzymatic catalysts and phase transfer catalysis. Various phase transfer catalysts were employed to catalyze the acylation of coenzyme A (CoA) with acetic anhydride. The acetyl-CoA was utilized by citrate synthase to form 964 eq of citric acid for each coenzyme A equivalent added to the reaction. This method offers the potential for preparing many different acyl-CoA derivatives of potential use as substrates for enzyme-catalyzed synthesis. The method opens up the possibility for synthesizing derivatives of natural products from simple acid anhydrides.

In the past few decades, chemists increasingly have employed enzymes as catalysts for performing various synthetic transformations. Recently, enzyme-catalyzed synthesis has shown promise for generating useful quantities of substances that cannot be prepared conveniently using traditional chemical methods (*1*). Enzymes provide many advantages in synthesis; they are usually stereoselective and stereospecific, work at ambient temperature and pressure, and operate in aqueous solution.

Most previous work using enzymes has employed hydrolytic and redox enzymes. Little effort has been directed toward using enzymes that catalyze carbon–carbon bond formation. This situation is due in part to the lack of commercially available enzymes that catalyze these reactions but is also due to the cofactor requirement of the more interesting carbon–carbon bond forming enzymes. Coenzyme A (CoA) thioesters are essential substrates for enzymes that catalyze carbon–carbon bond formation in fatty acid and polyketide biosynthesis (*2*). Chemical synthesis of these thioesters has been employed previously but is impractical due to the high cost of using stoichiometric amounts of coenzyme A (*3*). Compounds such as citric acid and acetyl-L-carnitine have been synthesized recently by recycling CoA and acetyl-CoA (*4*). This approach uses an enzyme-catalyzed recycling scheme and reduces the cost of acetyl-CoA requiring enzymes. The scheme is restricted by the limited substrate specificity of acetyl-CoA synthetase (*5*).

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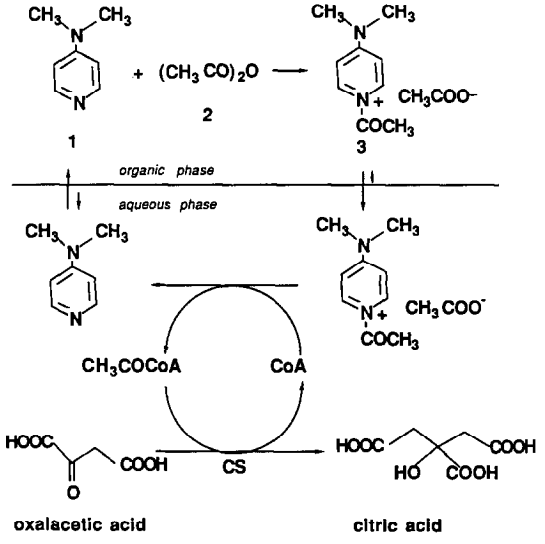
Enzyme-catalyzed reactions in multiphase systems are of substantial interest because they allow H_2O-insoluble substrates to be employed. Furthermore dramatic changes in the direction of equilibrium often are observed due to the reduced concentration of product in water. Various reactions have been performed using enzymes in organic solvents (*6*, *7*). In this paper, we report the first use of a phase transfer catalyst with an enzyme in a two-phase system. Phase transfer catalysts have been used to shuttle acylating groups between an aqueous and an organic phase. In this process an ionic intermediate is formed that is transported to the aqueous phase for reaction. This method previously has been named inverse phase transfer catalysis (IPTC) (*8*, *9*). In this paper we demonstrate the application of the IPTC process to an enzyme-catalyzed reaction system, providing a new way to recycle CoA and acetyl-CoA.

The overall reaction sequence is shown in Scheme 1. The catalyst dimethylaminopyridine (DMAP) **1** is acylated with acetic anhydride **2** to form intermediate **3.** **3** is ionic and transfers to the aqueous phase where acyl transfer to coenzyme A occurs liberating DMAP which returns to the organic phase for subsequent acylation. Acetyl-CoA transfers its acetyl group in an enzyme-catalyzed process releasing CoA which accepts another acetyl group from regenerated **3.** As a model system for this scheme we examined the enzyme-catalyzed aldol condensation between acetyl-CoA and oxaloacetate to form citric acid using citrate synthase.

In order to find the best conditions for this reaction, we examined various buffer systems, organic solvents, catalysts, and amounts of CoA and enzyme. The results are shown in Table 1. The total turnover number (TTN) in Table 1 is the



SCHEME 1. Acylation with enzyme-catalyzed system using phase transfer catalysis for CoA recycling.

TABLE 1

The Result of Enzyme Catalysis in a Two-Phase Reaction

Expt	Oxalacetic acid (mmol)	Ac_2O (mmol)	CS (U)	Solvent	CoA (mg, mmol)	Buffer
1	1.51	1.91	80	H_2O/CH_2Cl_2	10, 0.013	K_2HPO_4
1	1.51	1.91	80	H_2O/CH_2Cl_2	10, 0.013	Tris
2	1.41	1.91	80	H_2O/CH_2Cl_2	5, 6.5×10^{-3}	Tris
2	1.41	1.91	80	H_2O/CH_2Cl_2	5, 6.5×10^{-3}	Tris
3	1.40	2.63	80	H_2O/CH_2Cl_2	5, 6.5×10^{-3}	Tris
3	1.40	2.63	80	Toluene/H_2O	5, 6.5×10^{-3}	Tris
4	1.23	2.82	80	Toluene/H_2O	5, 6.5×10^{-3}	Tris
4	1.23	2.82	80	Toluene/H_2O	5, 6.5×10^{-3}	Tris
5	1.37	2.35	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
5	1.37	2.35	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
6	1.37	2.78	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
6	1.37	2.78	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
7	1.37	3.07	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
7	1.37	3.07	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
8	1.58	3.17	40	Toluene/H_2O	1, 1.3×10^{-3}	Tris
8	1.58	3.17	80	Toluene/H_2O	1, 1.3×10^{-3}	Tris
9	1.58	3.17	20	Toluene/H_2O	0.5, 6.5×10^{-4}	Tris
9	1.58	3.17	40	Toluene/H_2O	0.5, 6.5×10^{-4}	Tris
10	4.66	5.24	80	Toluene/H_2O	2, 2.6×10^{-3}	Tris
10	4.66	5.24	80	Toluene/H_2O	1, 1.3×10^{-3}	Tris
11	5.04	19.7	280	Toluene/H_2O	1, 1.3×10^{-3}	Tris
11	5.04	19.7	280	Toluene/H_2O	1, 1.3×10^{-3}	Tris

	Catalyst (mg)	Citric acid (mmol)	TTN
1	DMAP(6)	0.22	17 (yellow)
1	DMAP(6)	0.78	59
2	DMAP(6)	0.49	75
2	No	0.13	21
3	DMAP(6)	0.63	96
3	DMAP(6)	0.91	138
4	DMAP(6)	0.68	104
4	TMP(6)	0.18	27 (yellow)
5	DMAP(9)	0.83	324
5	DMAP(6)	0.70	267
6	DMAP(6)	0.79	304
6	No	0.20	78
7	DMAP(6)	0.73	282
7	TMP(6)	0.25	96
8	DMAP(10)	0.53	451
8	DMAP(10)	0.59	408
9	DMAP(10)	0.16	244
9	DMAP(10)	0.21	328
10	DMAP(10)	0.96	371
10	DMAP(10)	0.88	675
11	DMAP(10)	1.25	964
11	No	0.37	284

number of equivalents of citric acid produced per CoA equivalent added to the reaction. All the parameters examined in Table 1 are shown to affect the TTN. Tris buffer consistently gave higher TTNs than phosphate buffer because the latter contains Na^+ and K^+ ions which inhibit citrate synthase. Furthermore the choice of organic solvent is critical to high TTNs—toluene is consistently better than methylene chloride. This result is probably due to the higher solubility of methylene chloride in water causing the enzyme to lose activity. The choice of phase transfer catalyst is also important, with TTNs following the order: DMAP > 4-thiomethylpyridine (TMP) > no catalyst. Catalyzed reactions consistently produced three to four times higher total turnover numbers. With DMAP catalysis, 263 mg of citric acid was synthesized with a TTN of 964. Additional CoA did not produce higher amounts of citric acid suggesting that CoA is not the limiting reagent.

Coenzyme A, the most expensive component in this reaction ($400,000/kg), can be used in catalytic quantities as an acyl transfer reagent using inexpensive acetic anhydride as an acetylating reagent. This reaction is not limited by the substrate specificity of acetyl-CoA synthetase (*4, 5*). It offers the potential for preparing many different acyl-CoA derivatives for use as substrates for enzyme-catalyzed synthesis. As an example, we have synthesized fluorocitric acid from oxalacetic acid and fluoroacetyl CoA using citrate synthase by the IPTC process. In this procedure, fluoroacetyl chloride and DMAP are used as the acylating reagent and the catalyst, respectively. The TTN is only 56 because fluoroacetyl chloride is hydrolyzed more easily in water than acetic anhydride. In a different reaction, carnitine acetyltransferase was used as the acyl-CoA utilizing enzyme. In a control experiment, this enzyme denatured completely in a toluene/water mixture after only 2 h. Under these same conditions, citrate synthase does not lose activity after 4 h.

A limitation of this approach is the hydrolytic instability of acetic anhydride in contact with water. Another limitation is that some enzymes, such as carnitine acetyltransferase, gradually lose activity when added to two-phase systems. This loss of activity is attributable to the organic solvent-induced enzyme denaturation.

In summary, we successfully applied the IPTC process to an enzyme-catalyzed reaction system. This creates a new method for recycling CoA and acyl-CoA. It offers the potential for preparing many different acyl-CoA derivatives for use as substrates for enzyme-catalyzed synthesis. Furthermore, it opens up the possibility for using this method to synthesize derivatives of natural products starting from simple acyl anhydrides.

EXPERIMENTAL SECTION

The product (citric acid) was identified by ^1H NMR and fluorocitric acid was identified by ^1H NMR and ^{19}F NMR, using a 300-MHz Bruker Model AM300 instrument. The amount of citric acid was determined by using absolute ethanol as an internal standard. The amount of fluorocitric acid was determined by using fluorotribromomethane and 2,2,2-trifluoroethanol as the external standards with

D_2O as solvent. Oxalacetic acid, acetic anhydride, DMAP, and Tris were purchased from Aldrich. Citrate synthase and coenzyme A were purchased from Sigma. Fluoroacetyl chloride was purchased from Alfa.

The experiment was conducted in a 25-ml three-necked pear-shaped flask. A pH electrode was placed in the middle neck. Oxalacetic acid was dissolved in 2 M Tris base solution, pH 7.4. CoA was dissolved in the oxalacetic acid solution, and the catalyst was dissolved in the organic solvent. The two phases were stirred with a magnetic stir bar. Citrate synthase was added to the aqueous phase. Acetic anhydride was added into the organic layer by a syringe pump (120 μl/h), oxalacetic acid solution (70–80 mg) was added every hour, and citrate synthase (20 U) was added every 1–2 h. The pH of the reaction solution was kept between 7 and 8 with a pH controller by adding 2 M Tris base solution. The reaction was allowed to proceed between 4 and 17 h. The organic layer was removed and the precipitate of denatured enzyme was removed by filtration. Water was removed by lyophilization to afford a white powder.

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