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Intracellular Concentrations of Coenzyme A and Its Derivatives from *Clostridium acetobutylicum* ATCC 824 and Their Roles in Enzyme Regulation

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Intracellular levels of coenzyme A (CoA) and its derivatives involved in the metabolic pathways for *Clostridium acetobutylicum* ATCC 824 were analyzed by using reverse-phase high-performance liquid chromatography (HPLC). During the shift from the acidogenic to the solventogenic or stationary growth phase, the concentration of butyryl-CoA increased rapidly and the concentrations of free CoA and acetyl-CoA decreased. These changes were accompanied by a rapid increase of the solvent pathway enzyme activity and a decrease of the acid pathway enzyme activity. Assays with several non-solvent-producing mutant strains were also carried out. Upon entry of the mutant strains to the stationary phase, the butyryl-CoA concentrations for these mutant strains were comparable to those for the wild type even though the mutants were deficient in solvent-producing enzymes. Levels of acetoacetyl-CoA, β-hydroxy-butyryl-CoA, and crotonyl-CoA compounds in both wild-type and mutant extracts were below HPLC detection thresholds (<21 μM).

The anaerobic spore-forming bacterium *Clostridium acetobutylicum* is well known as a biological producer of acetone, ethanol, and butanol, all solvents of industrial interest (16). In batch culture, the typical acetone-butanol fermentation can be divided into two distinctive phases. In the acidogenic phase, the organism grows rapidly and produces acetate and butyrate, with the decrease in the pH of the medium resulting from the acid accumulation. In the solventogenic phase, the organism grows slowly and produces acetone, butanol, and ethanol, with the pH in the medium increasing slightly because of the acid reassimilation (6, 10, 24). The shift to solvent production is associated with the induction of solventogenic enzymes and a decrease in the activity of acidogenic enzymes (1, 10).

Although many investigators (7, 21, 22, 27, 32) have studied the biochemistry of acetone-butanol fermentation, the detailed mechanisms which control the shift to the solvent formation are not well understood. Significant progress on the analysis of the enzyme activities and internal pH involved in the clostridial pathway has been reported (4, 8–15, 17, 18, 20, 25–31). However, only recently have the in vivo levels of intermediate metabolites and cofactors, which may play an important role in enzyme regulation, been studied (9).

From the metabolic pathways related to the solvent-producing clostridia, it is clear that different coenzyme A (CoA) derivatives (acetyl-CoA [Ac-CoA], acetoacetyl-CoA [AcAc-CoA], β-hydroxyl-butyryl-CoA, crotonyl-CoA, and butyryl-CoA [Bu-CoA]) are key intermediates in the metabolism of this organism. The metabolic fate of these compounds may directly affect the product pattern of the fermentation (4, 5, 16). Reported here are the intracellular concentrations of free CoA and various CoA derivatives during the entire fermentation process, measured by using a reverse-phase chromatographic system. Assays on several non-solvent-producing mutant cultures for comparison were also carried out. Furthermore, it was of interest to study the correlations of CoA compound concentrations with the specific enzyme activity levels before and during the onset of solventogenesis.

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MATERIALS AND METHODS

Reagents. High-performance liquid chromatography (HPLC)-grade acetonitrile was purchased from EM Science (Gibbstown, N.J.), and sodium phosphate was from Mallinckrodt, Inc. (Paris, Ky.). Deionized water was further purified by passing it through a Milli-Q water purification system (Millipore Corp., Bedford, Mass.). CoA and its derivatives were obtained from Sigma Chemical Co. (St. Louis, Mo.). The dye-binding protein assay kit was obtained from Bio-Rad Laboratories (Richmond, Calif.)

HPLC apparatus. Separations of CoA compounds were performed with an LDC/Milton Roy GM 4000 HPLC system (Riviera Beach, Fla.). Two model III pumps were used to deliver eluants through a 5-μm octyldecyl silane column (4.6 by 250 mm) from Custom LC, Inc. (Houston, Tex.). A model 1203 UV-III detector was employed to monitor column effluent at 254 nm. The signal from the UV monitor was delivered to a CI-10B integrator for quantification.

Chromatography. The two mobile-phase solvents used were buffer A (0.2 M sodium phosphate [pH 5.0]) and buffer B (800 ml of 0.25 M sodium phosphate [pH 5.0] mixed with 200 ml of acetonitrile) (19). These buffers were passed through glass microfiber GF/F filters (Whatman International Ltd., Maidstone, England) prior to use. The chromatographic separations were performed at room temperature at a flow rate of 1 ml/min. The mobile-phase composition profile, after a 2.5-min constant buffer B level of 3% (i.e., 97% buffer A), was divided into several linear-gradient segments, with sequential segment end points of 7.5 min (where 18% buffer B was reached), 10 min (28% buffer B), 15 min (30% buffer B), 25 min (40% buffer B), 26 min (42% buffer B), and 35 min (90% buffer B). At the 90% buffer B level, the percentage of buffer B was reduced, over 1 min,

to the original 3% level and then maintained to the end of the HPLC cycle (45 min). Data collection was stopped at 40 min, and column reequilibration was complete after 47 min. A constant injection volume of 10 μl was maintained for all HPLC runs.

CoA and the various acyl-CoA derivatives were made up as 5 mM stock solutions in a 100 mM sodium phosphate buffer (pH 3.0) and stored at −80°C. Stock solutions prepared in this way were stable for at least a month.

Strains and culture conditions. The wild-type strain was *C. acetobutylicum* ATCC 824. Mutants 2BBD, M5, and 2BBR were isolated and described previously by Clark et al (3). Mutants 34, 4145, 1013, and 6a5 were Tn*916* mutants prepared as described previously by Sass and Bennett (23). Maintenance of these organisms, including preparation of the medium, inoculation, heat shocking, and anaerobic growth, have been described previously (3, 23).

Method of culture preparation. Strains were grown anaerobically in 600 ml of reinforced soluble medium (RSM) (3) at 37°C. The pH was not controlled. Samples were taken at 2- to 4-h intervals to obtain data corresponding to the maximal solvent-producing stage. The appropriate harvest time was determined initially by measuring pH and A_{600}. Samples were centrifuged at 19,000 × *g* for 20 min at 4°C, the supernatants were analyzed for solvent production by the nitroblue tetrazolium method (3), and the pellets were resuspended in the cold soluble medium and centrifuged at 34,000 × *g* for another 20 min at 4°C. No significant loss of metabolite levels was observed with this second step, which allowed better chromatographic separations. The pellets were immediately powdered in a porcelain mortar and pestle cooled to −80°C with liquid nitrogen. The frozen powder was stored at −80°C until required.

Methods of enzyme assays. Cell extracts were prepared as described previously (3) except that the protein concentration was measured by the method of Bradford (2) with bovine serum albumin as the standard. Measurement of each enzyme activity was performed as described previously (3).

Methods of CoA compound extraction and recovery. Frozen powder (0.4 g) was weighed into a polystyrene centrifuge tube precooled with liquid nitrogen, and 0.8 ml of cold 6% perchloric acid (PCA) containing 2 mM dithiothreitol was added. The mixture was sonicated at 90% power for 9 min for acidogenic cells and 15 to 18 min for solventogenic cells. The denatured protein was removed by centrifugation, and 0.8 ml of the PCA extract was transferred to a precooled Eppendorf centrifuge tube and adjusted to a pH of 3.0 by dropwise addition of 3 M K_2CO_3 with vortexing during the addition. The neutralized sample was centrifuged at 4°C, and the supernatant was filtered through a 10,000 NMWL ultrafree-MC filter (Millipore Products Division, Bedford, Mass.) The resulting aliquot was stored at −20°C when not immediately analyzed by using the HPLC. Solutions stored in this manner were stable for a week or more.

Recovery of added standard CoA compounds was accomplished as follows. Either a 100-μl standard CoA mixture or the 100 mM NaH_2PO_4 (pH 3.0) solution was added to a centrifuge tube and cooled to liquid-nitrogen temperature. A 0.4-g amount of frozen powder was then added to the tube and extracted with 0.8 ml of ice-cold 6% PCA as described above. The resulting aliquot was analyzed in the HPLC. Each sample was run at least twice in the HPLC.

Four different samples from wild-type *Clostridium* cultures were extracted, and the recoveries were calculated. The percent recovery for free CoA was found to be a mean of 95% with a ±3% standard deviation; the other recoveries were 97% ± 18% for Ac-CoA, 81% ± 6% for AcAc-CoA, 93% ± 2% for β-OH-butyryl-CoA, 82% ± 3% for crotonyl-CoA, and 89% ± 13% for Bu-CoA.

Determination of biomass concentration. Biomass concentration of the culture broth was measured by reading the optical density at 600 nm (OD_{600}) and converting the readings to concentrations in grams (wet weight) per liter by using a mass extinction coefficient of 0.89 ± 0.03 liter g^{-1} cm^{-1}. The mass extinction coefficient was determined by measuring the actual biomass concentration and the corresponding OD_{600} under various conditions.

Intracellular and extracellular aqueous space measurement. To measure the intracellular volume at various times during the batch fermentation, a 5-ml cell suspension was incubated anaerobically in a 15-ml disposable tube at 37°C in the incubator, with 1 μCi of [^{14}C-carboxyl]dextran (American Radiolabeled Chemicals Inc., St. Louis, Mo.) and 10 μCi of ^3H_2O (New England Nuclear, Boston, Mass.) added to the assay. Dextran was used for the measurement of the extracellular water volume of the pellets and had a final concentration of 0.142 g/liter and a radioactivity of 1.4 mCi/g. Tritium water (i.e., ^3H_2O) was used for measuring the total aqueous space in the pellet and had a final concentration of 110.7 mM (18.0 mCi/mol). The intracellular aqueous volume for the growing cells in their growth medium was determined by subtracting the extracellular space occupied by dextran from the total aqueous space (13, 14, 25). On the basis of 12 independent measurements each, the intracellular aqueous volumes of the acid-phase, solvent-phase, and late-solvent-phase cells are 0.21 ± 0.01, 0.36 ± 0.02, and 0.45 ± 0.02 ml/g (wet cell), respectively.

Calculations. Radioisotopes were used to measure the total (V_t), intracellular (V_i), and extracellular (V_o) aqueous volumes of the cell pellets. All units were milliliters per gram (wet weight).

PCA in the amount of 0.8 ml was added to 0.4 g of frozen cell powder (1:2 [wt/vol]). After extraction, the total aqueous volume for the CoA-compound solutions dissolved was 0.4 V_t + 0.8, expressed in milliliters.

After centrifugation, neutralization, and filtration to separate out unwanted cell debris, the aliquot was analyzed by HPLC while a 10-μl injection volume was maintained. The total amount of a measured CoA compound was determined from the expression $C_n(0.8 + 0.4\ V_t)/0.4$ g, or $C_n(2 + V_t)$, expressed in nanomoles per gram (wet weight), where C_n is the concentration of a specific CoA compound, which was derived from the HPLC. The intracellular concentration is therefore $C_n(2 + V_t)/V_i$, expressed in micromolar.

RESULTS

Chromatography. A standard solution of all of the CoA compounds involved in the clostridial pathway was separated to a useful degree in 40 min by reverse-phase chromatography (Fig. 1A). CoA and its derivatives were distinctly separated while maintaining the baseline approximately level, thereby allowing for more accurate quantification of CoA compounds.

The typical chromatograms of PCA extract from a *C. acetobutylicum* wild-type solventogenic culture and a mutant culture are shown in Fig. 1B and C. Peaks were identified through their retention times and through spiking by adding corresponding standards. Peak areas were plotted against concentration for each CoA compound for quantification. CoA, Ac-CoA, and Bu-CoA were well separated and found in measurable quantities. AcAc-CoA, β-OH-butyryl-

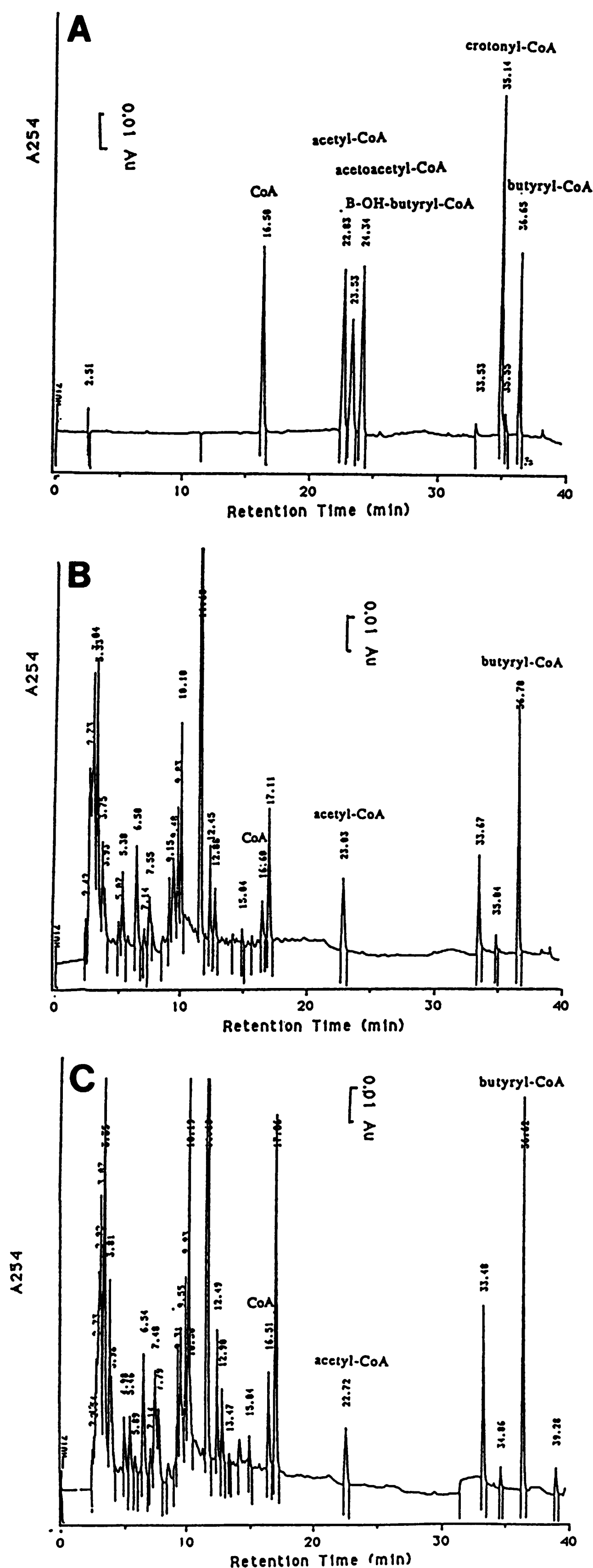

FIG. 1. (A) Elution profile of a mixture containing 1 nmol of CoA and noted acyl-CoA intermediate standards (detector sensitivity, 0.128 full-scale absorbance); (B) elution profile of a 10-μl PCA extract from wild-type *Clostridium* solventogenic cells; (C) elution profile of a 10-μl PCA extract from *Clostridium* mutant culture M5.

CoA, and crotonyl-CoA were separated in standard solutions but have not been found in measurable amounts in the cell extract.

Linearity, detection limits, and recovery. Linear relationships between the ratios of the peak areas of CoA compounds and the amounts added in the range of from 25 to 4,000 pmol were observed by using full-scale absorbance of 0.016 to 0.128. The respective lower limits of detection for CoA, A-CoA, AcAc-CoA, β-OH-butyryl-CoA, crotonyl-CoA, and Bu-CoA were 15, 15, 25, 15, 12.5, and 15 pmol (with corresponding intracellular concentrations of 11, 11, 21, 11, 10, and 12 μM for solvent-phase cells) at a minimum observed signal-to-noise ratio of 3.5.

Levels of intracellular CoA compounds from wild-type cultures. The intracellular CoA compound levels from a wild-type pH-uncontrolled batch culture were measured, with the results shown in Table 1. The cultures were harvested more frequently during the onset of the solventogenic phase so that the rapidly changing metabolic levels could be more clearly delineated.

It is known that the enzyme profiles of acid-phase and solvent-phase cells are different. The enzymes needed for acid production, detected from their catalytic activity in cell extracts, decrease in solvent-phase cells. Solvent-producing enzyme activity, in contrast, is present only in cells harvested during the solvent phase (1, 10). To further characterize the correlation of enzyme induction with intracellular concentrations of key intermediates, the enzyme activity and the corresponding CoA compound concentrations around the onset of solventogenesis were measured. These results are also shown in Table 1.

Intracellular levels of CoA compounds from mutant cultures. To form a better understanding of the relationship between the CoA compound concentrations and solventogenesis, various non-solvent-producing mutants isolated previously in this laboratory (3, 23) were analyzed. These mutants either did not produce any solvents (for mutants 6a5, 1013, M5, and 4145) or produced smaller amounts of solvents than did the wild-type strains (for mutants 2BBD and 34). Except for mutant 34, all mutants are non-spore-forming strains. These mutants either lack enzymes or have only low levels of the enzymes involved in the solvent production. The CoA compound concentrations for these mutants and the corresponding solvent pathway enzyme activities are listed in Table 2. Cultures were judiciously harvested during the early stationary phase. Cultures harvested past this point were difficult to sonicate and therefore could lose their enzyme activity quickly; the CoA compound levels would also decrease rapidly.

DISCUSSION

The production of solvents by using clostridia is well known, but the initiation mechanisms for the switch to solvent production are still being widely studied. Current hypotheses proposed for the triggering mechanism that cause the shift from the acid to the solvent production phase involve the regulation of catalytic pathways in response to the cellular concentrations of intermediates (5, 8). Gottwald

TABLE 1. Intracellular CoA compound concentrations and the solvent pathway enzyme activities in a pH-uncontrolled batch culture of wild-type *C. acetobutylicum*

Sample	OD_{600}	pH	Time (h)	Concn				Enzyme activity[a]						
				Butanol (mM)	CoA (μM)	Ac-CoA (μM)	Bu-CoA (μM)	PTB	BK[b]	NADH-BDH	NADPH-BDH	NAD-BAD	NADP-BAD	CoA transferase
Acid cells	2.40	4.27	11	0	185 ± 16	436 ± 22	574 ± 39							
Late acid cells	4.47	3.89	15	4.0	161 ± 14	456 ± 14	512 ± 18	20,100	900	5.7	22.5	14.6	12.6	17.9
Early solvent cells	5.12	3.82	16.5	21	124 ± 7	316 ± 9	810 ± 24	16,500	840	16.6	50.0	30.4	47.0	316
Solvent cells	5.14	3.82	18	>40	102 ± 3	327 ± 11	857 ± 23	12,400	680	4.8	47.7	22.3	22.3	613
	5.86	3.79	20		145 ± 6	396 ± 19	882 ± 30	13,200	600	7.8	85.7	51.6	34.4	1,200
	6.13	3.80	22		122 ± 10	327 ± 5	1,002 ± 19							
	6.26	3.93	23		154 ± 12	383 ± 21	690 ± 25							
	7.82	3.92	27		140 ± 1	368 ± 11	645 ± 21							

[a] Units for each enzyme are defined as nanomoles of product formed per milligram of protein.
[b] BK, butyrate kinase.

and Gottschalk (8) point out that, at the end of the acidogenic phase, where the reactions which lead from Bu-CoA via butyryl-phosphate to butyrate are reversible, an elevated concentration of butyrate will result in higher levels of butyryl-phosphate and Bu-CoA accumulating in the cell. This will result in significant decreases in both the CoA and the phosphate pools, triggering activation or synthesis of the enzymes for solventogenesis. Results herein show that, during the switch to solvent production, the Bu-CoA concentration exhibited a marked increase from 512 to 810 μM. Correspondingly, the CoA concentration decreased from 161 to 124 μM, and the Ac-CoA concentration decreased from 456 to 316 μM. In other stages of the fermentation, this concentration remains relatively constant (Table 1).

Recent data from Grupe and Gottschalk (9), who used a continuous culture of *C. acetobutylicum* DSM 1731, show that, during the shift, contrary to the expected positive pattern (first increase and then decrease) for Ac-CoA and Bu-CoA, a negative (first decrease and then increase) pattern for CoA, Ac-CoA, and Bu-CoA was observed. Results herein showed a similar negative pattern of CoA and Ac-CoA compared with their results. However, during the shift, a consistent increase in Bu-CoA concentration was detected, until the highest level of 1,000 μM was observed (Table 1). This discrepancy may result from the differences in the mode of the culture growth (continuous versus batch) and the culture operation. The batch cultures are in less-steady-state conditions than continuous cultures; therefore, some transient effect may not be observed.

To further characterize the correlation of solvent pathway enzyme induction and intracellular concentration of key intermediates, representative enzyme activities and CoA compound concentrations were also measured relative to the shift to the solventogenic phase (Table 1). These data clearly show that, along with the rapid increase in Bu-CoA concentration, the activity level of solvent pathway enzymes, namely CoA transferase, butyraldehyde dehydrogenase (BAD), and butanol dehydrogenase (BDH), also increase rapidly. Conversely, the activity of the acid-producing enzymes, phosphotransbutyrylase (PTB) and butyrate kinase decreased.

As a control, the results from a wild-type culture were compared with those of various mutant cultures at the onset of the stationary phase. These mutants are either non-solvent-producing strains (1013, 6a5, 4145, and M5) or are reduced-solvent-producing strains (2BBD and 34) because they lack the solvent-producing enzymes mentioned above. From these results (Table 2), it can be seen that the mutants generally exhibit reduced BDH levels and no CoA transferase or BAD activity. However, these mutants can produce Bu-CoA concentrations comparable to, or even higher, than those for wild-type cultures, possibly because the higher levels of butyrate cannot be reassimilated.

The main advantage of knowing the concentrations of metabolic intermediates is that, after comparing the kinetic and physiological properties of the enzymes involved, the influence of the metabolite in regulating a specific reaction can be evaluated. Those enzymes which react with the CoA

TABLE 2. Intracellular CoA compound concentrations and the solvent pathway enzyme activities from various *Clostridium* mutants at the onset of the stationary phase

Strain	OD_{600}	pH	Concn				Enzyme activity[a]						
			Solvent (mM)	CoA (μM)	Ac-CoA (μM)	Bu-CoA (μM)	PTB	BK[b]	NADH-BDH	NADPH-BDH	NAD-BAD	NADP-BAD	CoA transferase
Wild type	5.12	3.82	21	124 ± 7	316 ± 9	810 ± 24	16,500	840	16.6	50.0	30.4	47.0	316
1013	5.28	3.88	0	136 ± 2	319 ± 20	1,180 ± 61	10,900	1,480	1.1	17.2	0	0	0
6a5	5.34	3.95	0	76.4 ± 14.2	193 ± 18	1,101 ± 42	22,600	813	0	20.8	0	0	0
M5	6.00	3.92	0	284 ± 12	222 ± 8	1,169 ± 34	9,600	1,190	1.6	83.1	0	0	0
2BBD	6.26	3.76	NT[c]	162 ± 2	398 ± 1	679 ± 3	9,300	1,020	0	26.0	NT	NT	NT
34	4.52	3.93	7.0	88.0 ± 6.9	302 ± 3	761 ± 51	12,900	1,380	4.4	35.7	1.5	1.5	79.2
4145	5.27	3.72	0	159 ± 4	225 ± 7	1,326 ± 16	14,900	1,170	21.2	27.0	28.4	34.0	39.0

[a] Units for each enzyme are defined as nanomoles of product formed per milligram of protein.
[b] BK, butyrate kinase.
[c] NT, not tested.

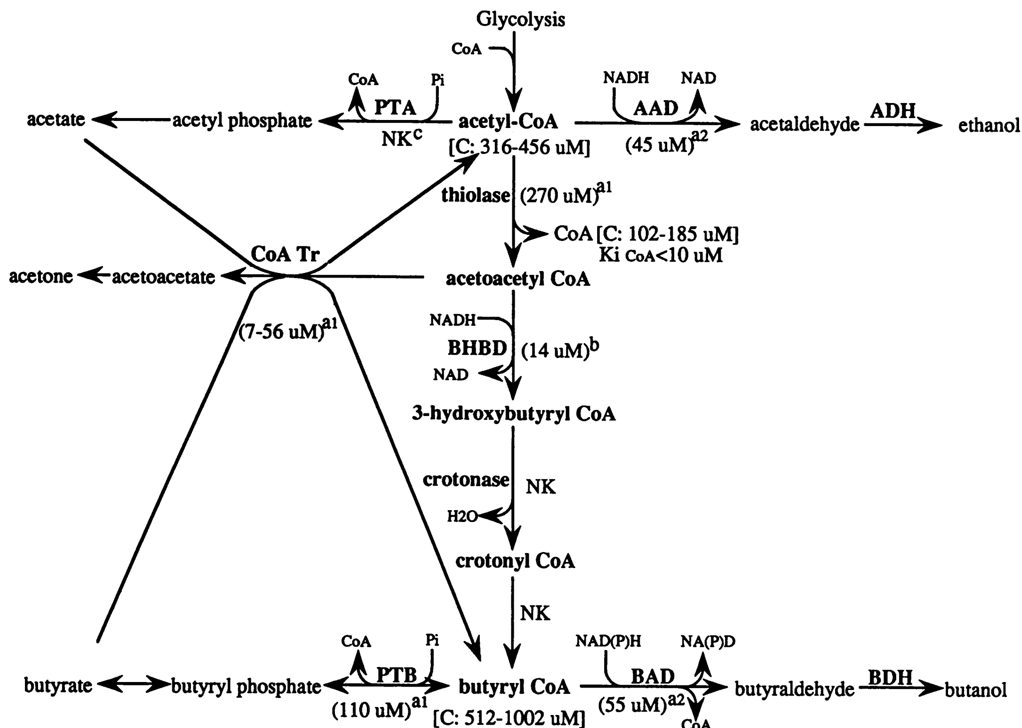

FIG. 2. Metabolic pathway of *C. acetobutylicum* and the kinetic properties of the enzymes. The K_m values are given in parentheses. The intracellular CoA, Ac-CoA, and Bu-CoA concentrations are given in brackets. Notes: [a], K_m values derived from *C. acetobutylicum* ATCC 824 ([a1]) and B643 ([a2]); [b], K_m values derived from *C. butylicum* NRRL B593; [c], NK, not known. Abbreviations: Ki, inhibition constant; PTA, phosphotransbutyrylase; AAD, acetaldehyde dehydrogenase; ADH, alcohol dehydrogenase; CoA Tr, CoA transferase.

derivatives are indicated in Fig. 2, along with the K_ms for the purified enzymes. Except for β-hydroxybutyryl-CoA dehydrogenase (BHBD), which was purified from *Clostridium butylicum* NRRL B593 (4), all other noted enzymes are derived from *C. acetobutylicum* (usually ATCC 824) (20, 27–30).

The measured intracellular concentrations of CoA, Ac-CoA, and Bu-CoA (Table 1) were generally above 100, 316, and 512 μM, respectively. These values are sufficient to allow maximal flux through the BAD and acetaldehyde dehydrogenase reactions for which the K_m values of 55 μM for Bu-CoA and 45 μM for Ac-CoA were determined (20). These values should also be able to nearly saturate the PTB reaction, for which a K_m value of 110 μM for Bu-CoA in the forward direction was reported (29). In fact, a change in internal pH may be an important factor in the regulation of the PTB reaction (29). Within the physiological pH change from 5.5 to 7, the internal pH range for typical fermentations (12), the PTB activity decreases significantly in the forward direction as pH decreases and shows virtually no activity below a pH of 6 (29). Therefore, the level of Bu-CoA is not seen as an important regulating factor for PTB activity, as suggested previously (29).

In the condensation reaction with thiolase, the K_m value for Ac-CoA had been determined to be 270 μM, and the thiolase activity was found to be inhibited by even micromolar levels of free CoA (28). Under these conditions, the intracellular CoA levels should be very inhibitory to this reaction, and the Ac-CoA would still be below a saturating level. The intracellular concentrations of CoA and Ac-CoA are likely important factors in regulating the net condensation of Ac-CoA to AcAc-CoA and in regulating the pattern of product formation.

Three of the CoA derivatives involved in the metabolic pathways, namely, AcAc-CoA, β-OH-butyryl-CoA, and crotonyl-CoA, were not detected. It was known that these compounds are unstable, and thus several extractions with externally added CoA compounds were carried out to check the reproducibility and recoverability of the extraction and assay procedures. From four different samples, the recoveries of AcAc-CoA, β-OH-butyryl-CoA, and crotonyl-CoA were 81, 93, and 82%, respectively. On the basis of these recoveries, the corresponding intracellular concentrations of AcAc-CoA, β-OH-butyryl-CoA, and crotonyl-CoA should be less than 21, 11, and 10 μM. The enzymes which react with these CoA derivatives, except Bu-CoA dehydrogenase, have been purified from either *C. acetobutylicum* (i.e., CoA transferase and crotonase [27, 30]) or *C. butylicum* (i.e., BHBD [4]), and the K_m values are shown in Fig. 2. In the crotonase reaction, the K_m value for the product crotonyl-CoA was 30 μM, and the enzyme was reported to be sensitive to levels of crotonyl-CoA above those observed here. However, the K_m value for β-OH-butyryl-CoA was not determined (27). For the BHBD from *C. butylicum*, a K_m value for AcAc-CoA of 14 μM was determined (4). This reference also reported that inhibition of BHBD activity by AcAc-CoA was observed at concentrations at or above its K_m when the NADH concentration was near its K_m, suggesting that AcAc-CoA plays an important role in the in vivo regulation of BHBD.

None of the three CoA intermediates could be detected when the mutant cultures were used (Table 2). Even in the

absence of solvent pathway enzymes, these compounds do not accumulate, thus suggesting a tight regulation of their synthesis. Further investigation of the role of these three CoA derivatives in enzyme regulation awaits the isolation of mutants having specific enzymes removed.

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