Lactate Reduction in Clostridium propionicum

PURIFICATION AND PROPERTIES OF LACTYL-CoA DEHYDRATASE*

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Clostridium propionicum converts lactate to propionate (Cardon, B. P., and Barker, R. A. (1947) Arch. Biochem. Biophys. 12, 165-171). We have obtained a soluble system that carries out this conversion as well as the hydration of acrylate to lactate and the reduction of acrylate to propionate. 3-Pentynyl-CoA inhibits reduction of acrylate and lactate to propionate, but not hydration of acrylate to lactate by cell extracts. The conversion probably involves CoA esters. When [3-2H] lactate is used as a substrate, the rate of propionate formation is reduced 1.8-fold, and the methyl group of the resulting propionate has lost 1.4 deuterium atoms. These results are consistent with the intermediate formation of acrylate (acrylyl-CoA) in the conversion of n-lactate to propionate.

Two proteins, which we designate E I and E II, were purified to >99% homogeneity. Together, they catalyze the hydration of acrylyl-CoA to lactyl-CoA. E I has an apparent molecular mass of 27,000 daltons and is rapidly and irreversibly inactivated by O2. E II consists of two subunits of molecular mass 41,000 and 48,000 daltons and contains equal amounts of riboflavin and flavin mononucleotide. Hydration of acrylyl-CoA to lactyl-CoA requires Mg** and catalytic quantities of ATP. GTP can replace ATP, but ADP and adenylyl imidodiphosphate cannot. We were unable to detect any stable intermediate during acrylyl-CoA hydration. Finally, we proposed a mechanism for this reaction.

Clostridia are obligate anaerobes that obtain energy via the Stickland reaction, which is shown in Scheme 1 (1). The bacteria are able to use the amino acid as both electron donor and electron acceptor. Clostridium propionicum, the organism of interest, converts alanine to lactic acid and then reduces it to propionate (2).

There are two known pathways for lactate reduction, the randomizing and the nonrandomizing pathways. In the randomizing pathway such as in propionibacteria, lactate is oxidized to pyruvate, which then condenses with CO2 to form oxalacetate (2). This is reduced and dehydrated to form succinate, which is converted to propionate via methylmalonyl-CoA. Since succinate is symmetrical, the a-carbon of lactate is converted equally to the a-carbon and b-carbon of propionate. During nonrandomizing or direct reduction of lactate as in Megasphaera elsenii and C. propionicum, label placed in the b-carbon of lactate ends up solely in the b-carbon of propionate (3). These organisms do not ferment any of the intermediates of the randomizing pathway to propionate nor do they require CO2 for lactate reduction (4).

In 1947, it was demonstrated that whole cells of C. propionicum rapidly ferment acrylate, as well as either lactate or alanine, to acetate and propionate (5). It was proposed that the lactate reduction involves acrylate as an intermediate, shown in Scheme 2.

It was later shown that C. propionicum contains a propionyl-CoA dehydrogenase which can catalyze the second half of Scheme 2; therefore, the reactions in Scheme 2 were proposed to occur at the level of CoA thioesters (2). Further evidence for the occurrence of acrylyl-CoA was provided by the purification of acrylyl-CoA aminase (acrylyl-CoA + NH2 = a-alanyl-CoA) (6). However, no evidence for the conversion of acrylyl-CoA to either lactate or alanine was obtained. Recently, it was found that in whole cells of C. propionicum incubated with lactate and 3-butynoic acid, which when activated to 3-butylnyl-CoA is an inactivator of acyl-CoA dehydrogenase, small amounts of acrylate accumulate (7). However, it was not clearly shown that acrylate was derived from lactate (7). A partial purification of a lactyl-CoA dehydrogenase from M. elsdenii was reported, although the activity detected was extremely low and dehydration could not be demonstrated (8). Thus, there is no conclusive evidence for the dehydratase of lactate (lactyl-CoA) to acrylyl-CoA.

In other clostridia, however, there is more convincing evidence for the dehydration of an a-hydroxy acid to the a,b-unsaturated acid. In a cell-free extract of Clostridium microsporum, the accumulation of [5-14C]glutaconate from [R-14C]2-hydroxyglutarate was demonstrated (9). Clostridium sporogenes accumulate small amounts of cinnamate during fermentation of 3-phenyl lactate (1). In both organisms, nothing is known about the mechanism of these dehydrations.

The dehydrations of lactate, 2-hydroxyglutarate, and 3-phenyl lactate are unusual elimination reactions. The hydrogen which is eliminated is not activated, and the hydroxy group is a very poor leaving group. This is in contrast to the majority of biological elimination reactions where the proton removed is a to a carbonyl group or other activating group and the leaving nucleophile is the carbonyl group. We now report the properties of the enzymes systems isolated from C. propionicum which catalyzes the dehydration of lactyl-CoA.

EXPERIMENTAL PROCEDURES

Materials—All materials were reagent grade or better. Acetyl phosphate, CoA, ATP, ADP, and AMP were from Sigma. L-[14C]Lactate was purchased from New England Nuclear, and DL-[14C]lactate was from Amersham Corp. Pantethine was purchased from Calbiochem-Behring and was reduced to pantetheine by a standard procedure (10).

Cell Growth and Media—C. propionicum (American Type Culture
Collection 25322) were stored in solid agar containing 3 g of alanine, 3 g of Bacto-peptone, 29 g of Difco thiglycolate media, 15 g of agar, 2.5 ml of saturated CaSO₄, 50 mg of MgSO₄, 20 mg of FeSO₄, and 1 mg of methylene blue in 1 liter of 10 mM potassium phosphate, pH 7.5. Cells were transferred from agar to 15 ml of the above medium minus the agar and grown at 37 °C. Cells were grown sequentially to 125 ml of flasks, and 10 liters in a previously described medium (5) with 1 mg liter⁻¹ methylene blue and 0.03% sodium sulfide added. Four liters of cells were used to inoculate 100 liters of medium. After sterilizing the medium, air was purged from the fermenter with N₂. The cells were harvested in a Delaval continuous flow centrifuge. A. This was centrifuged for 20 min at 25,000 × g. Frozen cells (10-15 g) were resuspended in 50 mM potassium phosphate, pH 7.5, 0.06% Na₂S₂O₄, and 1 mg liter⁻¹ methylene blue served as an indicator of anaerobiosis.

After harvesting, the cells were washed once with 50 mM potassium phosphate, pH 7.5, 0.06% Na₂S₂O₄, and 1 mg liter⁻¹ methylene blue. This was loaded onto a Sephadex G-150 column (91 × 2.5 cm) equilibrated with Buffer B in the same manner as E II was loaded onto the Sepharose 6B column. The column was eluted with Buffer B and fractions collected.

The enzyme required for acrylate hydration in Fraction I will be referred to as E I. Fractions containing E I were located as described for the DE32 column. These were pooled, and loaded onto a hydroxyapatite column (Bio-Rad DNA grade, 2 × 5 cm) equilibrated with Buffer B. Proteins were eluted with a 300-ml gradient of 0-200 mM potassium phosphate, pH 7.5, 0.06% Na₂S₂O₄, and 1 mg liter⁻¹ methylene blue. To each 10-ml fraction, 1 g of methylene blue was added. Fractions containing E I were located as above and pooled.

Protein Concentration—Protein concentrations were measured by the method of Bradford (11) with bovine albumin as a standard.

UV-visible Spectroscopy—Spectra were obtained with a PerkinElmer UV-1 spectrophotometer. HPLC—Three HPLC systems were used. HPX-87H Organic Acids Column (Bio-Rad) was used with a precolumn (5 × 0.3 cm) containing Aminex Q-15 S resin (Bio-Rad) and with a mobile phase of 5 mM H₂SO₄. The effluent was monitored with a Waters Refractive Index detector. The C₄₅ (Waters) and PRP (Hamilton) reverse phase columns were used with mobile phases as noted. CoA thioesters and flavins were detected with a Beckman UV detector at 254 nm.

Thin Layer Chromatography—Silica Gel G plates (without fluorescent indicator) were used with the following solvents: BuOH/HOAc/H₂O (12:38:5) (system A) and BuOH/CH₃OH/H₂O (7:2:1) (system B) (13, 14). System C was 5% NaHPO₄ on cellulose plates. Compounds were detected by their fluorescence under UV light.

Enzyme Assays (Fraction A.S.)—Lactate reduction was measured by incubating Fraction A.S. with 10 μmol of dl-[^14C]lactate (approximately 10,000 cpm μmol⁻¹), 10.8 μmol of acetyl phosphate, 10 μmol of MgSO₄, 4 μg of Na₂S₂O₄, and 0.5 μmol of CoA in a volume of 1.5 ml of 50 mM potassium phosphate, pH 7.5, and 1 mg liter⁻¹ methylene blue. After 16 min at 37 °C, the reaction was terminated by the addition of 25 μl of concentrated HClO₄, and the precipitate removed by centrifugation. Lactate and propionate were separated by HPLC on an HPX-87H column, and the amount of propionate formed was determined either by refractive index or by measuring radioactivity in lactate and propionate. Radioactivity was used only when at least 0.5 μmol of propionate was formed.

Acrylate reduction was measured by incubating Fraction A.S. with 10 μmol of sodium acetate, 0.5 μmol of CoA, 10.8 μmol of acetyl phosphate, and 4 μg of Na₂S₂O₄ in a volume of 1.5 ml of 50 mM potassium phosphate, pH 7.5, and 1 mg liter⁻¹ methylene blue. After 16 min at 37 °C, 20 μl of concentrated HClO₄ were added, and the precipitate was removed by centrifugation. Acrylate and propionate were separated by HPLC on the HPX-87H column, and the amount of propionate formed was determined by refractive index.

Acrylate hydration by Fraction A.S. was measured in assays identical to those for lactate reduction except the lactate was replaced with 20 μmol of sodium acrylate and the Na₂S₂O₄ halved. After 30 min, Na₂S₂O₄ was added to a final concentration of 0.05% and stored in the anaerobic chamber.

Fraction I was further purified as follows: Fraction I was dialyzed for 12 h against Buffer B and then loaded onto a small DE32 column (2 × 2 cm) equilibrated with Buffer B. The protein was eluted with 7.5 ml of 0.5 M potassium phosphate, pH 7.5, 0.06% Na₂S₂O₄, and 1 mg liter⁻¹ methylene blue. This was loaded onto a Sepharex G-150 column (91 × 2.5 cm) equilibrated with Buffer B in the same manner as E II was loaded onto the Sepharose 6B column. The column was eluted with Buffer B and fractions collected.

The enzyme required for acrylate hydration in Fraction I will be referred to as E I. Fractions containing E I were located as described for the DE32 column. These were pooled, and loaded onto a hydroxyapatite column (Bio-Rad DNA grade, 2 × 5 cm) equilibrated with Buffer B. Proteins were eluted with a 300-ml gradient of 0-200 mM potassium phosphate, pH 7.5, 0.06% Na₂S₂O₄, and 1 mg liter⁻¹ methylene blue. To each 10-ml fraction, 1 g of methylene blue was added. Fractions containing E I were located as above and pooled.

Protein Concentration—Protein concentrations were measured by the method of Bradford (11) with bovine albumin as a standard.

Determination of Lactate—Lactate was measured colorimetrically as described (12), except the centrifugation was omitted. 4-Phenyl-1-(2-thiazolinone)thiazolium bromide (Fluor) was recrystallized from EtOH before use.

Radioactivity—Radioactivity was measured using either a Beckman LS 100C or LS 1800 scintillation counter. Samples were dissolved in Amersham ACS scintillation fluid.

The abbreviations used are: FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; ADF, FNP, adenosyl-imidodiphosphate.

Scheme 1. Stickland reaction.

Scheme 2. Lactate reduction.
removal of the precipitate, the amount of lactate formed was determined colorimetrically.

**Identification of Propionate**—Propionate formed from lactate by Fraction A:S. under standard assay conditions was isolated directly by HPLC on an HPX-87H column. The fractions containing propionate were adjusted to pH 7 with KOH, and the solution was reduced under pressure. The precipitate was dissolved in 1 ml of H2O and concentrated HClO4 added to pH <1. The precipitate was removed by centrifugation and the propionate reisolated by HPLC. Fractions containing propionate were adjusted to pH 7 with KOH and lyophilized to dryness. The residue was taken up in 1 ml of HzO and concentrated HC104. Propionate was isolated and the NMR spectrum determined at 90 MHz.

**Synthesis of CoA Thioesters—Acetyl-CoA was synthesized from acetic anhydride by a modification of the procedure of Ochoa (15). Acetyl-CoA was synthesized from 3-pentynyl pantetheine (10). 3-Pentynoic acid (30 mg, 30.6 pmol) was added. This was twice passed through a CIS Sep-Pak equilibrated similarly to the acetyl-CoA. 3-Pentynyl-CoA was determined with Ellman's reagent (16).

3-Lactyl-CoA was synthesized using β-propiolactone (17) and purified identically to the acetyl-CoA. 3-Pentynyl-CoA was synthesized by a modification of a previously described procedure for the synthesis of 3-pentynyl pantetheine (10). 3-Pentynoic acid (30 mg, 30.6 µmol) (a kind gift of G. Fendrich) was dissolved in 1 ml of acetone and acetyl-CoA was added. After stirring for 30 min at 0°C, 20 mg of CoA dissolved in 2 ml of 0.2 M KHCO3, were added, and this was stirred for an additional 10 min. This solution was adjusted to pH 3 with HI and the precipitate removed under reduced pressure. This solution 0.5 ml of 1 M KC1 was added, the 3-pentynyl-CoA purified identically to the acetyl-CoA. The concentration of 3-pentynyl-CoA was determined with Ellman's reagent (16).

Acrylic-CoA was synthesized by a modification of a previously described procedure (16). CoA (20 mg) was dissolved in 2 ml of 0.2 M KHCO3 at 0°C, and 65 µl of acrylyl chloride were added with rapid stirring. After 5 min, the solution was titrated to approximately pH 3 with 1.0 M K2CO3. To the acrylyl-CoA solution, 1 ml of 1 M KC1 was added. This was twice passed through a CIS Sep-Pak equilibrated with 1 ml of HI and the eluent saved. The Sep-Pak was washed with 5 ml of 1 M HI to remove KCl and acrylyl-CoA was added. After stirring for 30 min at 0°C, 20 mg of CoA dissolved in 2 ml of 0.2 M KHCO3, were added, and this was stirred for an additional 10 min. This solution was adjusted to pH 3 with HI and the acrylyl-CoA used within 6 h. A large excess of acrylyl-CoA was used to enrich lactate to CoA in order to conserve material. The acrylyl-CoA will be referred to as Fraction B. After washing the Sep-Pak with MeOH and re-equilibrating it with 1 ml of HI, the acrylyl-CoA in Fraction A was purified as above, except only the 25% MeOH was saved and combined with Fraction B. ME0H was removed under reduced pressure, and the acrylyl-CoA used within 6 h. A large excess of acrylyl chloride to CoA was used in order to maximize the rate of formation of acrylyl-CoA, thereby minimizing the amount of CoA present with the acrylyl-CoA, as thiols rapidly react with acrylyl thioesters in a Michaelis addition (2).

The purity of acrylyl-CoA, the hydroxamates of acrylyl-CoA and methyl acrylate were chromatographed on Whatman No. 3MM paper developed with H2O/n-butyl alcohol, 18:100 (8). Hydroxamates were formed by the reaction of a large excess of neutral NH4OH (1:1 3.5 M NH4OH/14% NaOH) with either acrylyl-CoA or methyl acrylate. Acrylyl-CoA yielded two hydroxamates: Rf = 0.22 (major) and methyl acrylate yielded one hydroxamate, Rf = 0.22.

The concentration of acrylyl-CoA was estimated by determining its absorbance at 260 nm and assuming the ε260 of acrylyl-CoA and of crotonyl-CoA (ε260 = 22,800 M-1 cm-1) (18) are identical. The amount of acrylyl-CoA formed by this method represents an upper limit on the amount of acrylyl-CoA actually present, due to the instability of acrylyl-CoA.

**Synthesis of [2- and 3-3H]Lactates—[3-3H]Lactate was synthesized from [3-3H]pyruvate (19). Sodium pyruvate (0.55 g, 5 mmol) was dissolved in 10 ml of 0.06 M NaOH. After 48 h at room temperature, a 1H NMR spectrum showed only the 3H resonance. NaBH4 (1 g, 27 mmol) was added, and after 30 min at room temperature, the excess borohydride was destroyed with acetone. Borate esters were destroyed as described previously (20). Lactate was purified by chromatography on a Dowex-1 Cl column (1 × 18 cm). The lactate solution was loaded onto the column and eluted with 100 ml of H2O. Lactate was eluted with 50 mM HCl and concentrated by rotary evaporation. Lactate was further purified by HPLC on the HPX-87H column. The 1H NMR spectrum of the [3-3H]lactate was a singlet at 4.2 ppm. There was no detectable signal from any β-hydrogens. [3-3H]Lactate was synthesized and purified identically. For the specific activity studies, [3C,3-3H] and [4C,3-3H] lactates were prepared similarly. [2-3H]Lactate was prepared similarly to the [3-3H]lactate. To 0.5 g of sodium pyruvate in H2O, 1 g of NaBH4 was added. After 30 min, excess NaBH4 was destroyed with acetone, and the lactate was purified identically to the [3-3H]lactate. The 1H NMR spectrum (D2O) was a singlet at 1.47 ppm, and there was no indication of any α-protons.

**NMR Experiments—[3-3H]Lactate (60 µmol) was incubated with Fraction A:S. (42 mg of protein), 4 mg of CoA, 30 µmol of MgSO4, 1 mg of ATP, 12 mg of Na2SO4, and 10 mg of acetyl phosphate in a final volume of 0.2 ml. The reaction was stopped by the addition of 50 mM potassium phosphate, pH 7.5, and 0.5 µmol of tritium labeled methyl acetate was added. After incubation for 30 min at 37°C, 50 µl of concentrated HClO4 were added. The precipitate was removed by centrifugation, and propionate was isolated by HPLC on the HPX-87H column. The propionate containing solution was titrated to pH 7 with KOH and lyophilized to dryness. To the residue 0.5 ml of 0.5 M KOH was added, and the sample was lyophilized to dryness again. After 0.8 ml of H2O was added, the NMR spectrum was determined at 90 MHz.

**Isolation of Lactyl-CoA**—The products of 10 acrylyl-CoA hydration assays as described in Table IV were chromatographed on the PRP reverse phase HPLC column with a mobile phase of 3.5% CH3CN in 10 mM ammonium acetate, pH 5.8. Fractions were collected and lactate detected colorimetrically (12). A portion of each fraction was dried at 100°C, and the residue was dissolved in 1.0 ml of 0.1% TFA and analyzed by HPLC. The lactyl-CoA was eluted with 3 ml of 50 mM potassium phosphate, pH 7.5, containing 1 mg liter-1 methylene blue. This was incubated for 20 min at 37°C and then the reaction was stopped with 50 µl of concentrated HClO4. Propionate was isolated and the NMR spectrum determined as above, except a 500 MHz NMR spectrometer was used. Gels—Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the procedure of Laemmli (21), with a 4% spacing and 10% running gel. Coomassie Brilliant blue was used to visualize proteins. Non-denaturing gel electrophoresis was performed similarly, except the sodium dodecyl sulfate and 2-mercaptoethanol were omitted. A 3% spacing gel and 7.5% running gel were used.

**Removal of Cofactors from E**—To prepare the lactyl-CoA for NMR spectroscopy, it was lyophilized to dryness and the residue dissolved in 2 ml of 1 M HI. This was lyophilized to dryness and the residue taken up in 0.75 ml of 1 M HI. The NMR spectrum of the lactyl-CoA upfield from HzO was determined at 90 MHz.

The lactyl-CoA was air-dried by incubating it for 30 min at 37°C with 0.1 N KOH and then acidifying to pH 3 with HCl. Ellman's reagent was used to determine the concentration of sulfhydryl groups (10).

To prepare the lactyl-CoA for NMR spectroscopy, it was lyophilized to dryness and the residue dissolved in 2 ml of 1 M HI. This was lyophilized to dryness and the residue taken up in 0.75 ml of 1 M HI. The NMR spectrum of the lactyl-CoA upfield from HzO was determined at 90 MHz.
the starting solvent and then a linear gradient to 100% MeOH over 72 min was applied. All HPLC of flavins and Factors I and II were done using this solvent system. Compounds of interest were collected, and the MeOH was removed by slightly warming the solution and blowing N\textsubscript{2} over the surface. For this and all subsequent procedures, exposure to light was minimized. The compounds were lyophilized to dryness and stored at -20 °C. Flavins were also removed by incubating E II for at least 24 h at room temperature in the presence of O\textsubscript{2}.

Three volumes of MeOH were added, and precipitated protein was removed by centrifugation. To prepare the supernatant fluid for HPLC, MeOH was removed by blowing a stream of N\textsubscript{2} over the surface of the solution.

Compounds were dissolved in 50 mM Tris-HCl, pH 8.1, prior to treatment with phosphodiesterase (Sigma, Crotalus Atrox) or alkaline phosphatase (Sigma, Type III R). Phosphodiesterase (0.2 mg) was added to <20 mM of the compound and incubated for 30 min at room temperature. If the compound was not going to be further treated with alkaline phosphatase, 2 volumes of MeOH were added and the sample chilled to 0 °C. To digest with alkaline phosphatase, 0.014 mg of alkaline phosphatase was added and incubated for 60 min at room temperature. Two volumes of MeOH were added, and the solution was chilled to 0 °C. To prepare samples for HPLC, the precipitated protein was removed by centrifugation and then MeOH removed with a stream of N\textsubscript{2}.

For UV-visible spectroscopy and KIO\textsubscript{3} cleavage, the compounds were dissolved in 50 mM ammonium acetate, pH 5.8. To cleave the sample with KIO\textsubscript{3}, a 7.5-fold molar excess of KIO\textsubscript{3} was added. The amount of Factors I and II was estimated by assuming \( \varepsilon_{325} = 32,500 \) M\textsuperscript{-1} cm\textsuperscript{-1}. The reaction was kept at room temperature for 20 min, and the products were purified by HPLC.

**RESULTS**

**Conversion of Lactate to Propionate and Reduction of Acrylate to Propionate in a Cell-free System**—Initial experiments were carried out in a cell-free system obtained from *C. propionicum* which has been subjected to (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} fractionation (Fraction A.S.). This system carries out the conversion of [\textsuperscript{14}C]lactate to [\textsuperscript{14}C]propionate (Table I). There is an absolute requirement for acetyl phosphate, CoA, and Mg\textsuperscript{2+}. ADP and ATP stimulate the reaction significantly, but AMP has no effect. ATP at concentrations up to 1 mM does not eliminate the requirement for acetyl phosphate. Pantetheine cannot replace CoA. \( \beta \)-Lactate was not reduced to propionate, nor was it converted to lactate. In this system, Na\textsubscript{2}SO\textsubscript{4} serves both as a reducing agent and also to keep the system anaerobic.

In the early stages of these experiments, it became apparent that the ability of Fraction A.S. to catalyze the conversion of lactate to propionate was rapidly lost upon exposure to air. The O\textsubscript{2} sensitivity of the enzyme system was further investigated. Aliquots of Fraction A.S. containing methylene blue were made aerobic by shaking until the dye became oxidized and then reduced with a small amount of Na\textsubscript{2}SO\textsubscript{4} at various times thereafter. The ability of these aliquots to reduce lactate to propionate was then assayed. After a 1-min exposure to oxygen, lactate reduction was inhibited >90%. This loss of catalytic activity is irreversible, since dialysis of Fraction A.S. inactivated by O\textsubscript{2} against Buffer B for 24 h at 4 °C did not restore activity.

The stereoisomer of lactate which is reduced to propionate is \( \beta \)-lactate, in accordance with previously reported results (7). When L-[\textsuperscript{14}C]lactate was added to standard assays containing 10 \( \mu \)mol DL-lactate, no radioabeled propionate was found, while when DL-[\textsuperscript{14}C]lactate was used [\textsuperscript{14}C]propionate was found.

Fraction A.S. also catalyzes the hydration of acrylate to lactate. The cofactor requirements for acrylate hydration were the same as for lactate reduction. Under standard assay conditions (see "Experimental Procedures"), Fraction A.S. hydrated 0.070 \( \mu \)mol of acrylate min\textsuperscript{-1} mg of protein\textsuperscript{-1}. To eliminate the possibility that the acrylate was first reduced to propionate and then converted to lactate, propionate was substituted for acrylate. There was no conversion of propionate to lactate. Thus, the lactate formed from acrylate was not formed via propionate. Exposure to O\textsubscript{2} for 1 min as described above also resulted in nearly complete inactivation of acrylate hydration.

Fraction A.S. catalyzes the rapid reduction of acrylate to propionate. Under standard assay conditions (see "Experimental Procedures"), Fraction A.S. reduced 1.72 \( \mu \)mol of acrylate min\textsuperscript{-1} mg of protein\textsuperscript{-1}. Na\textsubscript{2}SO\textsubscript{4} supplies reducing equivalents. ADP (0-2.8 mM), ATP (0-2.6 mM), and Mg\textsuperscript{2+} (0-33 mM) affected acrylate reduction 80%.

The ability of the extract to reduce acrylate to propionate was significantly less sensitive to O\textsubscript{2} exposure than the conversion of lactate to propionate or the hydration of acrylate. Exposure to O\textsubscript{2} for 10 min had no effect on the acrylate reductase activity. However, exposure of Fraction A.S. to O\textsubscript{2} for 24 h resulted in complete loss of the acrylate reductase activity.

**Effect of 3-Pentynyl-CoA—3-Pentynyl-CoA, a mechanism based inactivator of butyryl-CoA dehydrogenase, was examined as an inhibitor of acrylate reduction in fraction A.S.** The data in Fig. 1 show that the ability to convert acrylate to propionate is rapidly lost after addition of 3-pentynyl-CoA to Fraction A.S. Loss of activity is irreversible, since activity is not regained after 24-h dialysis against Buffer B.

To ascertain that the CoA thioester of 3-pentoynoic acid is the inactivating species, 2 mM 3-pentoynoic acid, 4.4 mM acetyl phosphate, and 0.4 mM CoA was incubated with Fraction A.S. at 37 °C for 30 min. Acrylate reduction was inhibited 18%, whereas incubation with 0.3 mM 3-pentoynyl-CoA resulted in complete inactivation. Therefore, 3-pentoynyl-CoA is probably the inactivating species, and Fraction A.S. does not efficiently catalyze the activation of 3-pentoynoic acid to the CoA thioester.

We also examined the effect of 3-pentoynyl-CoA on lactate reduction. While the inactivation of acrylate reduction was rapid, there was no inhibition of lactate reduction until >90% of the acrylate reduction was inactivated (Fig. 1). The rate of acrylate reduction was always greater than the rate of lactate reduction, consistent with acrylate being an intermediate in lactate reduction.

To ascertain that the inhibition of lactate reduction by 3-
phosphate, pH were as in Table I, except the concentration of lactate was not the same. The conversion to propionate was examined. Assay conditions for the conversion of acrylate to lactate were as described in Table I, except each assay contained 0.05 mg of O2-inactivated Fraction A.S. Standard acrylyl-CoA hydration assays as described in Table I were used to assay Fraction I (A), except the final volume was 2.0 ml. Protein concentration (O) was determined as described under "Experimental Procedures."

The effect of substitution of 1H in the β position of lactate on acrylate hydration was examined. Complete inactivation of acrylate hydration was induced by exposure to O2. Thus, initially, we attempted to find a fraction which could restore activity to Fraction A.S. inactivated with O2. The elution profile obtained when Fraction A.S. was chromatographed on DE32 is shown in Fig. 2. Addition of Fraction I to O2-inactivated Fraction A.S. restores the ability of the Fraction A.S. to convert acrylate to lactate. In assays as described in Fig. 2, 0.042 mg of Fraction I and 0.46 mg of O2-inactivated Fraction A.S. hydrated 1.57 pmol of acrylate min⁻¹ mg Fraction I⁻¹. Fraction I remains active.

The rate of acrylate hydration was examined by determining the rate of reduction of [3-'H3]lactate to propionate. While 43% of the acrylate remained, no [14C]acrylate was detected by HPLC (HPX-87H column). The rate of reduction of [3-'H3]lactate to propionate showed an isotope effect of 1.8 ± 0.05. An isotope effect of this magnitude is probably a primary isotope effect and suggests that in the conversion of lactate to propionate a C-H bond in the β position of lactate is broken. These results are, therefore, consistent with the intermediate involvement of acrylate or an ester of acrylate in the conversion of lactate to propionate.
for at least 2 months with little loss of activity when stored at 4 °C in the anaerobic chamber.

A second fraction, Fraction II, was found that when combined with Fraction I could hydrate acrylate to lactate (Fig. 2). However, in some preparations, the combination of Fraction I and Fraction II did not convert acrylate to lactate. We thought that acrylate was first activated to acrylyl-CoA, and that in some preparations the enzyme(s) required for acrylate activation were separated from the enzyme(s) that were responsible for hydration. This line of reasoning led us to try acrylyl-CoA as substrate. In the presence of ATP and Mg²⁺, the combination of Fractions I and II always converted acrylyl-CoA to lactate. As will be shown later, the actual product in lactyl-CoA.

Fraction II was purified as described under "Experimental Procedures." Purified Fraction II, E II, was stored at 4 °C in the anaerobic chamber in the presence of Na₂S₂O₄. The specific activity and yield of E II during the purification are shown in Table II. Under these conditions, E II remains active for at least 1 month. Purified E II migrates as a single peak upon chromatography on a Mono-Q FPLC column. Upon electrophoresis on polyacrylamide under nondenaturing conditions, a single band was detected. On a denaturing gel, however, two bands were detected (Fig. 3). The intensity of each band was measured with a densitometer. The amount of stain in each band was equi within 3%. The apparent molecular weights are 41,000 and 48,000. It thus appears likely that E II consists of two nonidentical subunits in a 1:1 ratio.

From denaturing gel electrophoresis, E II has a minimum molecular weight of 89,000. However, on a Sephadex G-150 column (molecular weight exclusion limit = 300,000), E II is completely excluded from the gel. On a Sepharose 6B column, Kᵥ for E II = 0.25, indicating a molecular weight of approximately 1 x 10⁶. Thus, E II is a multimer, although the exact molecular weight is unknown.

E II is a yellow protein. The spectrum of oxidized E II is shown in Fig. 4. It consists of the 280 nm protein absorbance and a broad plateau from 410 to 550 nm. The addition of 1 mM Na₂S₂O₄ reduces the absorbance by E II of wavelengths greater than 370 nm by 10%, although the shape of the spectrum does not change. The effect of Na₂S₂O₄ on the spectrum below 370 nm could not be determined due to the Na₂S₂O₄ absorbance. Reoxidation of the E II by shaking with air restored the original spectrum.

The time course of lactate formation from acrylyl-CoA catalyzed by Fraction I and E II was determined. The rate of acrylate hydration is nonlinear as, after 4 min, the rate was only 18% of the initial rate. This is probably due to: 1) a significant portion of the acrylyl-CoA is converted to lactyl-CoA (After 4 min, 22% of the acrylyl-CoA was hydrated) during the assay; 2) product inhibition by the lactyl-CoA; and 3) acrylyl-CoA is destroyed nonenzymatically via a Michaelis addition. The half-life of acrylyl-CoA under the assay conditions is approximately 90 s (data not shown).

Neither Fraction I nor E II alone can convert acrylyl-CoA to lactyl-CoA. To determine if either Fraction I or E II converts acrylyl-CoA to an intermediate that is subsequently converted to lactate, complete assays as described in Table II minus either Fraction I or E II, or both, were incubated at 37 °C for 5 min. The missing component(s) was added to each assay, and they were incubated for an additional 5 min. The amount of lactyl-CoA formed when either Fraction I or E II was initially omitted was less than or equal to the amount formed when both E II and Fraction I were omitted. The experiment was repeated, except the initial incubation was only 1 min, and the same results were obtained. Thus, neither

| Table II |
| --- |
| Purification of E II |
| E II was purified from 14 g of cells as described under "Experimental Procedures." E II was measured in standard acrylyl-CoA hydration assays containing 0.56 mM acrylyl-CoA, 0.17 mM CoA, 0.2 mg ml⁻¹ Sepharose 6B or 0.034 mg ml⁻¹ FPLC-purified E II. | |
| | Total Specific Total |
| | protein | activity | activity |
| | mg | μmol mg⁻¹ 5 min⁻¹ | μmol 5 min⁻¹ |
| DE32 | 113 | 0.27 | 30.5 |
| Sepharose 6B | 42 | 1.16 | 48.7 |
| FPLC | 20.8 | 3.13 | 65.1 |

**Fig. 3.** Denaturing gel electrophoresis of E I and E II. Electrophoresis of E I (a) and E II (b) was performed as described under "Experimental Procedures." The gel was stained and then scanned with a densitometer. The beginning and end of each gel are denoted by A and B.

**Fig. 4.** The absorption spectrum of E II. E II (1.2 mg ml⁻¹) was in 0.25 mM potassium phosphate, pH 7.5.
Lactyl-CoA Dehydratase

E II nor Fraction I converts acrylyl-CoA to a stable intermediate.

Fraction I was further purified as described under "Experimental Procedures" and stored in the anaerobic chamber at 4 °C. The purified protein will be referred to as E I. The specific activity and yield of E I during the purification are shown in Table III. The specific activity and total activity of the E I are lower limits, since the assay is not linear in E I, i.e., increasing the amount of E I 2-fold results in a less than 2-fold increase in the amount of lactyl-CoA formed. The amount of E I present in assays at each stage of purification was adjusted such that the same amount of lactyl-CoA was formed in each assay. Therefore, the relative specific activities at each stage are comparable. The results of denaturing gel electrophoresis of E I are shown in Fig. 3. The protein with an apparent molecular weight of 27,000 represents 90% of the protein in purified E I. Thus, it is likely E I consists of a single polypeptide with a molecular weight of 27,000.

The cofactor requirements for acrylyl-CoA hydration by E I and E II are shown in Table IV. ATP and Mg\(^{2+}\) are absolutely required. The amount of lactyl-CoA formed is greater than the amount of ATP added; therefore, stoichiometric ATP hydrolysis does not occur. While GTP can replace ATP, ADP, and ADP-PNP, a nonhydrolyzable ATP analogue cannot. Thus, it is likely that ATP hydrolysis is required to "activate" the system. Acrylyl-CoA is the actual substrate, since acrylate and CoA (± acetyl-CoA and acetyl phosphate) did not substitute for acrylyl-CoA. The addition of 0.26 mM CoA resulted in 22% inhibition of acrylyl-CoA hydration. β-Lactyl-CoA is not converted to lactate nor does it inhibit when added to the complete reaction mixture. β-Lactyl-CoA is, therefore, not an intermediate in the conversion of acrylyl-CoA to lactyl-CoA.

Identification of Cofactors Bound to E II—The cofactors bound to E II were removed by either precipitating the protein with HClO\(_4\) and then rapidly neutralizing the solution or by incubating E II for at least 24 h with O\(_2\) and then precipitating the protein with MeOH. After removing the precipitated material by centrifugation, the yellow supernatant fluid was chromatographed on the C\(_{18}\) HPLC column. Two UV-absorbing compounds designated Factor I and Factor II were detected.

Factor II from either MeOH or HClO\(_4\), precipitated E II had the same retention time as riboflavin on HPLC. The UV-visible spectrum of Factor II is identical to riboflavin. (A\(_{266}\), riboflavin = 266, 375, and 444 nm; A\(_{266}\) Factor II = 266, 373, and 443 nm). Factor II co-migrated with riboflavin on TLC systems A (R\(_S\) = 0.48) and B (R\(_S\) = 0.4). Treatment of Factor II with phosphodiesterase or alkaline phosphatase did not alter its retention on HPLC, suggesting that Factor II contains neither a phosphodiestere bond nor a terminal phosphate, as expected for riboflavin. To obtain further evidence that Factor II is riboflavin, both were treated with KIO\(_4\). After treatment with KIO\(_4\), both the product from Factor II and that derived from riboflavin co-chromatographed on HPLC. The UV-visible spectrum of each was identical (A\(_{266}\) = 442, 370, and 266 nm), and they co-migrated on TLC systems A (R\(_S\) = 0.61 (major), 0.71 (minor) and B (R\(_S\) = 0.65). These results establish that Factor II is riboflavin. Furthermore, it was found that under the conditions used to isolate Factor II, FMN and FAD are stable. Therefore, Factor II is not derived from FMN or FAD during the course of isolation.

Factor I from MeOH-precipitated E II had the same retention time as FMN on HPLC. Factor I has a UV-visible spectrum identical to FMN (A\(_{266}\), FMN = 266, 373, and 445 nm; A\(_{266}\) Factor I = 266, 372, and 444 nm) and co-chromatographed with FMN on TLC systems A and C. When Factor I was treated with alkaline phosphatase, the product co-chromatographed with riboflavin in HPLC and on TLC systems A and C, consistent with the identification of Factor I as FMN. Alkaline phosphatase-treated Factor I and riboflavin were incubated with KIO\(_4\). The product derived from Factor I co-chromatographed with the product obtained from riboflavin in HPLC and on TLC systems A and C. These results establish that Factor I is FMN.

The amount of FMN was equal to that of riboflavin as determined by comparing peak areas after HPLC. The absolute amount of FMN on E II was also estimated by HPLC. There were 0.5 mol of FMN (and, therefore, 0.5 mol of riboflavin) per mol of E II. This number represents a lower limit on the amount of flavin on E II because 1) O\(_2\) causes release of flavin from E II and E II is exposed to significant amounts of O\(_2\) during purification and 2) not all of the flavins may actually be liberated from E II.

Identification of Lactyl-CoA—Lactate, a product of acrylyl-CoA hydration by E I and E II, is measured colorimetrically. In this method, the lactate is first oxidatively decarboxylated to acetaldehyde under strongly acidic conditions and then the amount of acetaldehyde determined. Treatment of the assay products with base sufficient to hydrolyze any thioesters did not increase the amount of lactate detected colorimetrically. It is likely that under the assay conditions, lactyl-CoA can be detected.

| Reaction mixture | Lactate formed (nmol) |
|------------------|----------------------|
| Complete         | 640                  |
| - E I            | 5                    |
| - E II           | 0                    |
| - Acrylyl-CoA    | 0                    |
| - Mg\(^{2+}\)    | 75                   |
| - ATP (50 nmol)  | 614                  |
| - ATP            | 0                    |
| - ATP, + ADP (0.21 mmol) | 32 |
| - ATP, +ADP-PNP (0.30 mmol) | 3  |
| - ATP, +GTP (0.22 mmol) | 644 |
| - Acrylyl-CoA, +β-lactyl-CoA (0.68 mmol) | 0  |

† When E II was precipitated with HClO\(_4\), the retention time of Factor I was different from any of the standards and varied from 16 to 29 min. This material was not further investigated.
hydrolyzed to lactate so that the colorimetric assay does not
distinguish between lactyl-CoA and lactate. Therefore, the
product resulting from acrylyl-CoA hydration was examined
further. The products of acrylyl-CoA hydration assays (see
Table IV, Complete Reaction Mixture) were chromatographed
on the PRP HPLC column. The eluent was collected, neutralized,
and dried and then assayed for lactate colorimetrically.
Two peaks that gave a positive reaction in the colori-
metric assay for lactate were observed. The first, which co-
chromatographed with lactic acid, had a \( k' = 0.3 \) and con-
tained 11% of the apparent lactate. The second had a \( k' = 1.2 \)
and contained the remaining material that gave a positive
result in the colorimetric assay. This second peak was repu-
rified, again using the PRP column. The NMR spectrum of
this material represents the sum of the spectra of lactate and
CoA. No sulfhydryl groups were detected with 5,5'-di-
thiobis(2-nitrobenzoic acid) in the purified product, whereas
after base hydrolysis, the concentration of sulfhydryl groups
measured by 5,5'-dithiobis(2-nitrobenzoic acid) was equal to
the concentration of lactate measured colorimetrically. The
UV spectrum of the purified material was very similar to the
spectrum of acetyl-CoA. Thus, the product of acrylyl-CoA
hydration is lactyl-CoA, and the colorimetric assay for lactate
will measure lactyl-CoA and lactate equally well.

DISCUSSION

Cell-free extracts of C. propionicum catalyze the conversion
of lactate to propionate. Our results indicate that this conver-
sion proceeds through CoA thioesters and that acrylyl-CoA is
probably an intermediate (see equation below).

\[
\text{Propionate} \rightarrow \text{Acetyl-CoA} \rightarrow \text{Acrylyl-CoA} \rightarrow \text{Lactyl-CoA} \rightarrow \text{Propionate}
\]

The requirement for CoA and acetyl phosphate suggests the
presence of a CoA transferase system involved in the forma-
tion of lactyl-CoA. A CoA transferase from C. propionicum
that transfers CoA from acetyl-CoA to lactate, acrylate, or
propionate was recently isolated (22). When [3-\(^{3}H\)3]lactate
was used, an isotope effect was found in the conversion of

\[
\text{Lactate} \rightarrow \text{Lactyl-CoA} \rightarrow \text{Propionate}
\]

lactate to propionate, indicating that abstraction of a \( \beta-
hydrogen is partially rate-limiting. This is consistent with
intermediate formation of acrylyl-CoA and would not be
expected if lactyl-CoA was reduced directly to propionyl-CoA.
The existence of an acrylyl-CoA reductase was demonstrated,
and it was shown that inactivation of this enzyme abolishes
the reduction of lactate to propionate but not the hydration
of acrylyl-CoA to lactate. Finally, we have purified two enzymes
which together catalyze the conversion of acrylyl-CoA to
lactyl-CoA.

The enzyme system which converts lactyl-CoA to acryl-
CoA is surprisingly complex. It consists of two proteins which
we designated E I and E II. E II consists of two nonidentical
subunits, and E I consists of one subunit. The reaction re-
quires ATP in substoichiometric amounts. While nonhydro-
lyzable ATP analogues cannot replace ATP, GTP can. Thus,
it is likely that ATP hydrolysis is required. The mechanism
by which ATP activates the system is unknown, and we were
unable to demonstrate a time dependence of this activation
(data not shown). Our results differ from those reported
earlier (22) that ATP inhibited lactate reduction. This dis-
crepancy may be due to the greater purity of our preparations.
Furthermore, two flavins, riboflavin and FMN, are associated
with E II. This is the first enzyme we know of that uses
riboflavin.

The mechanism of the hydration is, for reasons pointed out
in the Introduction, of considerable interest, and the possible
role of the flavins and ATP is intriguing. It was proposed that
2-hydroxyglutarate dehydration involves an enzyme-bound
hydroxyl radical as shown in Scheme 3a (23). While the
involvement of a radical during dehydration of 2-hydroxycar-
boxylic acids is attractive, we think an enzyme-bound hy-
droxyl radical is highly unlikely. Instead, we propose the
mechanism shown in Scheme 3b. Initially, a \( \beta \)-hydrogen rad-
cal is abstracted. The resultant carbon radical combines with
a group M, which could be a highly reduced metal or a flavin.
The group M donates one electron and hydroxide is elimi-
nated. M is reduced to its original state by the initially
abstracted hydrogen. M is reduced to its original state by the initially
abstracted hydrogen. While we have no direct evidence for
this mechanism, a number of workers have demonstrated that
α-hydroxyl alkyl radicals in the presence of several reduced metals are converted to an alkene, hydroxide ion, and one electron-oxidized metal (24–29). The extreme O₂ sensitivity of E I may be due to the presence of a highly reduced metal or a radical. This point is now under investigation.

We have established that acrylyl-CoA is converted to lactyl-CoA, but we were unable to demonstrate the conversion of lactyl-CoA to acrylyl-CoA. This is also true in the crude system. Although this system converts lactate (lactyl-CoA) to propionate, no evidence for the intermediate formation of acrylate or acrylyl-CoA was obtained. This raises the possibility that free acrylyl-CoA is not formed. The lactyl-CoA dehydratase and acrylyl-CoA reductase might normally exist in a complex, such that the acrylyl-CoA is passed directly from the dehydratase to the reductase. Alternatively, one of the proteins required for lactyl-CoA dehydration may serve as a carrier protein for the acrylyl moiety. Thus, the actual intermediate in lactate reduction is an acrylyl enzyme which can either be hydrated to lactyl enzyme or reduced to propionyl enzyme. It would not be surprising if biological systems avoid the formation of free acrylyl-CoA due to its extreme reactivity towards nucleophiles.

While we have isolated the enzymes required for acrylyl-CoA hydration, the mechanism of this difficult reaction is not yet clear. Efforts are underway to elucidate this mechanism.

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REFERENCES
1. Bader, J., Rauschenbach, P., and Simon, H. (1982) FEBS Lett. 140, 67-72
2. Stadtman, E. R., and Vagelos, P. R. (1958) Proceedings of the International Symposium in Enzyme Chemistry, 1957, Tokyo and Kyoto, pp. 80-92, Maruzen, Tokyo
3. Leever, F. W., Wood, H. G., and Stjerneholm, R. (1955) J. Bacteriol. 70, 521-530
4. Johns, A. T. (1952) J. Gen. Microbiol. 6, 123-127
5. Cardon, B. P., and Barker, H. A. (1947) Arch. Biochem. Biophys. 12, 165-171
6. Vagelos, P. R., Earl, J. M., and Stadtman, E. R. (1969) J. Biol. Chem. 234, 490-497
7. Aledo, M., Cooney, C. L., and Sinskey, A. J. (1983) Bio/Tech. 1, 791-794
8. Baldwin, R. L., Wood, W. A., and Emery, R. S. (1965) Biochim. Biophys. Acta 97, 202-213
9. Buckel, W. C. (1980) Eur. J. Biochem. 106, 439-447
10. Gomes, B., Fendrich, G., and Abeles, R. H. (1981) Biophys. Acta 97, 202-213