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Phosphotransacetylase from *Clostridium acidiurici*[1]

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The phosphotransacetylase from *Clostridium acidiurici* has two properties not observed for this enzyme in other bacteria: (i) it requires a divalent metal for activity, and (ii) it is not subject to uncoupling by arsenate. The enzyme has been obtained in highly purified form, with a specific activity 500-fold higher than crude extracts. Ferrous or manganous ions are required for maximal activity, with Mn^{2+} being 50 to 75% as effective as Fe^{2+}. The acetyl group can be transferred from acetyl phosphate to coenzyme A in 20 mM arsenate without a net decrease in high-energy acyl linkages. Likewise, $H^{32}PO_4^{2-}$ will exchange with acetyl-PO_4^{2-} in the presence of arsenate without loss of acetyl phosphate. This suggests that the active site on the enzyme is capable of discriminating between phosphate and arsenate while permitting the reversible transfer of acyl groups between CoA and phosphate.

Clostridium acidiurici satisfies all of its carbon, nitrogen, and energy requirements by the fermentation of certain purines. Uric acid, xanthine, guanine, and hypoxanthine are degraded through a series of enzymatic steps to acetate, ammonia, and carbon dioxide, with the production of adenosine triphosphate (ATP). One important step in this degradative pathway involves the transfer of an acetyl group from acetyl-*S*-CoA to orthophosphate to form acetyl-PO_4. The acetyltransferase catalyzing this reaction is phosphotransacetylase (acetyl-*S*-CoA:orthophosphate acetyltransferase, EC 2.3.1).

Initial studies with protein fractions from *C. acidiurici* revealed that this enzyme possessed two properties not common to phosphotransacetylases from other sources; it required ferrous ions for activity and was not subject to arsenolysis (12).

This early report of a nonarsenolyzable phosphotransacetylase was regarded as an exception to the commonly observed property that these enzymes are subject to uncoupling by arsenate. Recently, however, Sucker and coworkers (3, 4, 20, 21) have shown that the phosphotransacetylases from the honey bee, housefly, leech, and yeast are also nonarsenolyzable, possibly indicating that generalizations in this regard are premature.

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None of the phosphotransacetylases previously described have shown an absolute requirement for metal ions at catalytic levels, although in high concentrations several ions such as NH_4^+, K^+, SO_4^{2-}, and PO_4^{2-} have been shown to effect enzyme activity (2, 7, 14). The relatively high concentrations of ions required in these cases for stimulation or stabilization suggest that they function predominantly in maintaining the tertiary structure of the enzyme rather than in a catalytic role.

Phosphotransacetylase from *C. acidiurici* has an absolute requirement for catalytic levels of ferrous ion and, analogous to the animal and plant systems investigated by Sucker, is not subject to arsenolysis. Previous data, supported by comparison with arsenolyzable systems, suggest that the ferrous ion requirement might render the catalytically active site capable of discriminating between orthophosphate and arsenate, thereby preventing arsenolysis.

The purpose of this investigation was to characterize the purified enzyme and continue the evaluation of its unusual properties.

MATERIALS AND METHODS

Cultivation of C. acidiurici. *C. acidiurici* was maintained in stock cultures, grown for enzyme preparation, and harvested as described by Sagers and Carter (13). The cells prepared in this manner were stored under a nitrogen atmosphere in tightly stoppered tubes at −20 C until used. Phosphotrans-

acetylase activity was stable in the frozen cells over a period of several months.

Peptococcus glycinophilus. Cells of *P. glycinophilus* were grown anaerobically at 37 C in a medium containing 0.5% glycine, 0.5% yeast extract, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, and 0.01% $Na_2S_2O_4$ as a reducing agent. Following centrifugation, the harvested cells were subjected to the same treatment listed below (through ammonium sulfate precipitation) for the preparation of cell-free extracts of *C. acidiurici*. The *P. glycinophilus* fraction precipitating between 60 and 70% saturation with ammonium sulfate was used in these studies without further purification. The protein content of the extract was 30 mg per ml.

Preparation of cell-free extracts. Wet-packed cells (50 g) of *C. acidiurici* were suspended in 100 ml of 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.0 (henceforth referred to as Tris buffer), containing 1 mM ethylenediaminetetraacetate (EDTA). The cells were ruptured by shaking the suspension for 6 min at 4 C with 150 g of 0.1-mm glass beads in the large cup of a Bühler cell homogenizer (RHO Scientific, Inc., Commack, N.Y.). The homogenate was centrifuged for 20 min at 16,000 × *g* to remove the glass beads and cell debris. The supernatant fluid and a Tris buffer wash of the pellet were subjected to a second centrifugation at 16,000 × *g* for 40 min. The supernatant fluid from the second centrifugation typically exhibited a 280/260 nm ratio of 0.62 to 0.65. The cell-free extract was diluted with Tris buffer to contain approximately 10 mg of protein per ml.

Protamine sulfate treatment. Tris buffer containing 1% protamine sulfate (Sigma Chemical Co., St. Louis, Mo.) was added dropwise with continuous stirring to the cell-free extract to achieve a final ratio of 10 ml of protamine sulfate solution per 100 ml of extract. After stirring for 20 min, the precipitated nucleic acids were collected by centrifugation for 20 min at 16,000 × *g* and discarded. The 280/260 nm ratio of the protamine-treated extract was typically 0.9 to 1.0.

Ammonium sulfate precipitation. Solid ammonium sulfate (special enzyme grade, Schwarz-Mann, Orangeburg, N.Y.) was added with continuous stirring to the protamine-treated extract to obtain fractions precipitating between 0 and 60, 60 and 80, and 80 and 100% of saturation. All fractions, except the one precipitating between 80 and 100%, were stirred for 20 min before collecting the precipitates by centrifugation for 20 min at 16,000 × *g*. The 80 to 100% fraction was allowed to stand undisturbed for 7 to 10 days at 0 C before collecting the precipitate by centrifugation. All fractions were redissolved in a minimal volume of Tris buffer.

Sephadex gel filtration. Sephadex G-100 columns were used for gel filtration. Dry Sephadex G-100 (10 to 40 nm bead size) was suspended in double-distilled water in a 2-liter vacuum flask and placed in a boiling water bath for 3 hr to completely rehydrate the gel. Fine particles were removed from the swollen gel by repeating a process of suspension in double-distilled water, allowing the gel to settle for 45 min, and then decanting the supernatant fluid containing the slow-sedimenting particles. The uniformly sedimenting gel was then suspended in enough Tris buffer containing 5 mM EDTA to give a gel (settle volume)-to-buffer ratio of 1.0. This suspension was brought to approximately 50 C and degassed under negative pressure. The suspension was then cooled to approximately 5 C before pouring into a glass column (either 4.5 by 90 cm or 2.5 by 60 cm). A conical funnel was fitted to the top of the column as a reservoir to allow the dilute gel suspension to be poured in one step. The gel was allowed to settle overnight under a head pressure of 10 cm of water to a final height of 50 cm of gel. The column was then equilibrated for 5 days with Tris buffer containing 1 mM EDTA. A Mariotte flask was used to maintain a constant head pressure such that the flow rate was 30 ml per hr. A gel column (2.5 by 50 cm) was equilibrated for 7 to 10 days with Tris buffer containing 0.1 mM EDTA and 0.2 M ammonium sulfate. This prolonged equilibration procedure was necessary to achieve a completely stabilized column capable of producing the repeatable sample elution patterns required in molecular weight analysis.

Ultrafiltration. Amicon model 12 and 202 ultrafiltration cells (Amicon Corp., Lexington, Mass.) were used with either PM-30 or PM-10 membranes to concentrate fractions containing phosphotransacetylase from both a Sephadex G-100 column (4.5 by 50 cm) and a subsequent diethylaminoethyl (DEAE)-cellulose column. A nitrogen pressure of 55 psi was used during ultrafiltration.

DEAE-cellulose chromatography. Thin-layer chromatography grade DEAE-cellulose (obtained from Sigma Chemical Co., St. Louis, Mo.) was used in these studies and was washed consecutively for 30 min with 0.2 N HCl, 0.2 N NaOH, and finally 1 M Tris buffer containing 10 mM EDTA. The slurry of exchanger was poured into a glass column (1.1 by 15 cm) and packed under 10 pounds of nitrogen pressure until a column bed (1.1 by 12 cm) was attained. The column was then placed at 5 C and equilibrated with 100 void volumes of the above buffer. Elution of proteins applied to the column was carried out stepwise with equilibration buffer containing increasing concentrations of KCl (0.0 to 0.6 M). Fractions of 2 ml each were collected at a constant flow rate of 10 ml per hr.

Preparation of acetyl-PO_4. The lithium salt of acetyl-PO_4 was prepared from isopropenyl acetate and phosphoric acid by the method of Stadtman and Lipmann (19), or purchased from Sigma Chemical Co., St. Louis, Mo.

Preparation and assay of pantetheine. Pantetheine was prepared from pantethine (Sigma) by a modification of the borohydride reduction procedure of Sagers, Benziman, and Klein (12). Sodium borohydride (400 mg) was added to 10 ml of absolute ethanol in which 2 g of pantethine had been dissolved. This reaction mixture was incubated at 2 to 3 C for 60 min. The ethanol was removed by vacuum distillation at 30 C and the residue was dissolved in 10 ml of double-distilled water. This aqueous solu-

tion was then saturated with ammonium sulfate (room temperature) and extracted three successive times with 10 ml of *n*-butanol. The butanol extracts were combined, and the butanol was removed by vacuum distillation at 40 C. The residue was redissolved in 10 ml of double-distilled water, and the pantetheine concentration in this preparation was determined by the 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) sulfhydryl assay method of Ellman (6). The pantetheine solution was then diluted to 0.2 M with water, distributed into tubes (2 ml each), and stored in the frozen state until needed.

Preparation of CoA solutions. One hundred milligrams of CoA (P.L. Biochemicals, Inc., Milwaukee, Wis.) was dissolved in 1 ml of double-distilled water and assayed for free —SH groups by the DTNB method of Ellman (6). The CoA preparation was then diluted with water to 0.2 M reduced CoA and stored in the frozen state until needed.

Protein determinations. Protein concentrations were determined by the method of Warburg and Christian (24) by using the ratio of the ultraviolet absorbancies at 280 and 260 nm.

Spectrophotometry. Spectrophotometric measurements were made by using either a Beckman DU or a Cary model 15 spectrophotometer.

Standard phosphotransacetylase assay procedure. The phosphotransacetylase from *C. acidiurici* was measured by detecting the generation of acetyl-*S*-pantetheine or acetyl-*S*-CoA from acetyl-PO_4 with substrate amounts of pantetheine or CoA (12). The concentrations of acetyl-PO_4, acetyl-*S*-pantetheine, and acetyl-*S*-CoA were determined by reacting them with hydroxylamine to form a stable acetyl-hydroxamic acid. Acetyl-hydroxamic acid was detected by the absorbance at 540 nm in an acidic $FeCl_2$ solution (10). Prior to incubation with substrate (acetyl-PO_4), the enzyme was activated for 5 min in 0.9 ml of solution containing (micromoles): Tris-hydrochloride, *p*H 8.0, 300; $FeSO_4 \cdot 7H_2O$, 2.0; and pantetheine or CoASH, 20.0. The enzyme activation and reaction with substrate were carried out at 37 C. The reaction was initiated by the addition of 20 μmoles of acetyl-PO_4 (0.1 ml) to the activation mixture. The reaction was allowed to proceed for 10 min before termination. For measurement of acetyl-*S*-CoA and acetyl-*S*-pantetheine, 1 ml of 0.2 N HCl was added, and this mixture was heated to boiling for 6 min and then rapidly cooled to room temperature. Neutralized 2 N NH_2OH (1 ml) was mixed with the boiled mixture and allowed to react for 10 min. Finally, 2 ml of 2% $FeCl_2$ in 1.3 N HCl was added to this solution. The contents of these tubes were then mixed thoroughly and centrifuged at 500 × *g* for 5 min to remove any protein precipitate. The absorbance was read at 540 nm against a nonenzyme blank.

When total acetyl groups (acetyl-PO_4 plus acetyl-*S*-pantetheine or acetyl-*S*-CoA) were to be measured, the reaction was terminated with 1 ml of 1 mM *p*-hydroxymercuribenzoate (PHMB), *p*H 9.0, since 0.2 N HCl plus boiling for 6 min destroys the acetyl-PO_4 present (18).

Definition of units and specific activity. One unit of activity is defined as that amount of enzyme required to catalyze the formation of 1 μmole of acetyl-*S*-pantetheine in 10 min using acetyl-PO_4 as the acetyl donor. Specific activity is defined as micromoles of acetyl-*S*-pantetheine formed per 10 min per milligram of protein.

Arsenolysis conditions. Phosphotransacetylases, in the presence of arsenate and catalytic levels of CoASH, have been shown to transfer acetyl groups from acetyl-PO_4, through an acetyl-*S*-CoA intermediate, to $HAsO_4^{2-}$ to form the unstable acetyl-AsO_4. The spontaneous hydrolysis of acetyl-AsO_4 is detected by the decrease in total high-energy acetyl groups measured as acetylhydroxamic acid (18). The conditions for enzyme activation and reaction with substrate are essentially the same as those in the standard phosphotransacetylase assay above except that 10 to 40 μmoles of potassium arsenate was added to the reaction mixture and the pantetheine or CoASH concentrations were reduced from 20 mM to 0.2 mM.

$H^{32}PO_4^{2-}$ exchange reaction. The exchange reaction catalyzed by phosphotransacetylase:

$$\text{Acetyl-PO}_4 + H^{32}PO_4^{2-} \rightleftharpoons \text{acetyl-}^{32}\text{PO}_4 + HPO_4^{2-}$$

was measured by separating acetyl-PO_4 from HPO_4^{2-} by using the selective $CaCl_2$ precipitation method of Lipmann and Tuttle (9).

Radioactivity of samples was measured in a Packard Tri-Carb liquid scintillation spectrometer. The scintillation fluid consisted of 0.7% 2,5-diphenyloxazole, 0.3% dimethyl-1,4-bis-2-(4-methyl-5-phenyloxazolylbenzene, and 10% naphthalene in freshly distilled reagent grade dioxane. The scintillation reagents were purchased from Packard Instrument Co., Downers Grove, Ill., and the $H_3{}^{32}PO_4$ was purchased from New England Nuclear Corp., Boston, Mass.

Marker proteins. Marker proteins for molecular-weight determinations were purchased from the following sources: cytochrome *c* (type III from horse heart), α-chymotrypsinogen (6 times crystallized, type II from bovine pancreas), deoxyribonuclease I (1 time crystallized and lyophilized), and bovine serum albumin (crystallized and lyophilized) from Sigma Chemical Co., St. Louis, Mo.; myoglobin (crystalline, lyophilized from sperm whale) from Schwarz-Mann, Orangeburg, N.Y.; ovalbumin (2 times crystallized) from Worthington Biochemical Corp., Freehold, N.J.; and hexokinase (lyophilized from yeast) from Nutritional Biochemical Co., Cleveland, Ohio.

Phosphotransacetylase preparation used in experiments. Except for the preparations shown in Tables 3 and 4, the enzyme from *C. acidiurici* used for experiments described in all other tables and figures was the purified (500-fold) protein from the DEAE-cellulose column described earlier. Larger quantities of enzyme were used when pantetheine was the acetyl group acceptor than when CoA was the acceptor.

RESULTS AND DISCUSSION

Purification summary. A summary of the purification procedures resulting in an increase in specific activity of the phosphotransacetylase activity of over 500-fold is shown in Table 1.

Electrophoresis. The enzyme purification procedures used were evaluated by polyacrylamide disc-gel electrophoresis (5). As shown in Fig. 1, the PM-10 concentrate from the DEAE-cellulose column contained a relatively homogeneous protein preparation with only minor contaminant components. The major protein component in this preparation was the only one associated with detectable phosphotransacetylase activity.

Heat stability of the enzyme. Samples of the purified enzyme in Tris buffer containing 1 mM EDTA and 0.2 M ammonium sulfate were heated at 88 C for periods of time up to 60 min. Compared with the activity of unheated samples, 56% of the activity remained in the sample heated for 60 min. This fact, coupled with the observation that negligible activity is lost after incubation for 60 min at temperatures lower than 80 C, indicates that the enzyme is relatively resistant to irreversible heat denaturation.

Absorption spectrum. The ultraviolet-visible absorption spectrum of the purified enzyme displayed a single peak at 280 nm, with no other detectable shoulders or peaks at other wavelengths which might indicate specific tightly bound prosthetic groups. This spectrum is similar to that observed for the phosphotransacetylase from *C. kluyveri* (2).

Molecular-weight determination. The molecular weight of the enzyme was estimated by gel filtration, as described by Andrews (1), using a column (2.5 by 50 cm) of Sephadex G-100, equilibrated and eluted with Tris buffer containing 0.2 M ammonium sulfate. Significant improvement in peak resolution was made possible by the presence of the ammonium sulfate. The elution volumes for phosphotransacetylase and the proteins used as standards, shown in Fig. 2, represent the average from three determinations.

The phosphotransacetylase activity was resolved into two peaks with estimated molecular weights of 75,000 and 63,000, respectively. No further attempts have been made to characterize the properties of the two peaks separated by this procedure, however.

The molecular weights determined for the *C. acidiurici* phosphotransacetylase (63,000 and 75,000) are intermediate between the only other reported values for this enzyme, which are those from *C. kluyveri* (38,000 to 41,000) and *Escherichia coli* B (160,000 to 450,000),

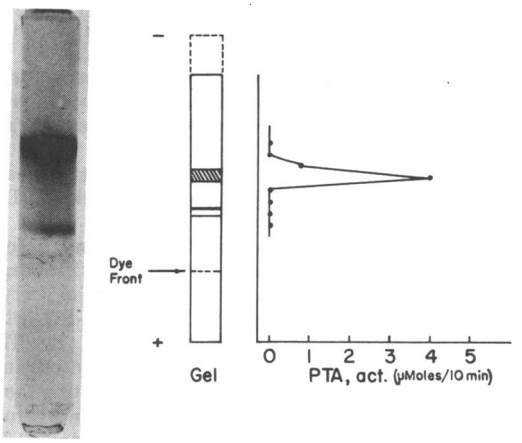

FIG. 1. *Recovery of the enzyme from disc electrophoresis gels. The purified protein (300 μg) was layered in 10% sucrose onto duplicate acrylamide gel columns (0.9 by 7.5 cm), consisting of a 3.5% stacking gel and a 7.5% running gel. Current was applied at 5 ma per gel for 1.5 hr while the gel tubes were kept at 5 C. The electrophoretic run was terminated after tracking dye (bromophenol blue) had traveled 5 cm into the running gel. The gels were removed from the tubes, and one gel was stained in 1% amido black to detect protein and then destained in 5% acetic acid at 100 ma per gel, whereas the other gel was cut into 3-mm sections which were forced through a syringe into standard pantetheine assay mixtures. PTA act. = phosphotransacetylase activity.*

TABLE 1. *Purification of phosphotransacetylase*

Procedures	Vol (ml)	Units (μmoles/ 10 min)	Protein (mg)	Specific activity (units/mg)	Recovery (%)	Fold purification
Cell-free extract	380	59,300	4,110.0	13.9	100.0	
Protamine sulfate	415	42,700	3,240.0	13.2	72.0	0.9
80 to 100% $(NH_4)_2SO_4$	11	57,500	320.0	178.0	97.0	12.8
Sephadex G-100	10	35,100	85.0	413.0	59.2	29.7
DEAE-cellulose	2	17,600	2.5	7,100.0	29.6	511.0

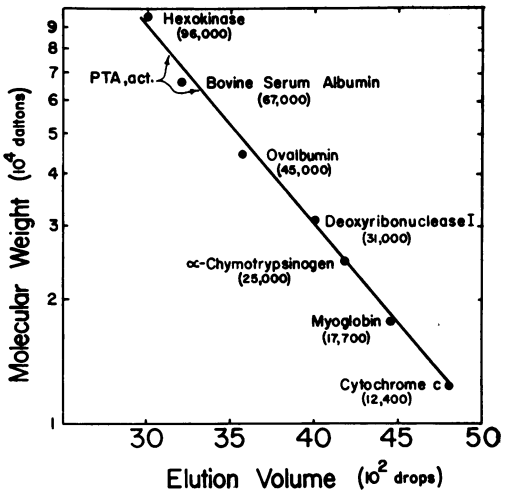

FIG. 2. *Molecular-weight determinations. Marker proteins and purified phosphotransacetylase were placed separately onto a column (2.5 by 50 cm) of Sephadex G-100. The column was equilibrated with Tris buffer containing 0.2 M ammonium sulfate at 5 C. Elution was carried out using the equilibration buffer at a flow rate of 10 ml per hr. Fractions of 40 drops each were collected and monitored at 280 nm for protein content. Fractions were tested for phosphotransacetylase activity when this enzyme was applied to the column.*

respectively (2, 14).

Effect of buffer systems and pH on enzyme activity. Substitution of barbital, borate, orthophosphate, or pyrophosphate for the standard Tris buffer system resulted in inhibition of the enzyme activity by 53, 25, 52, and 100%, respectively, whereas tricine or bicine gave 136 and 104% of the activity observed with Tris. The inhibition by orthophosphate will be considered in greater detail in a section following.

In Tris buffer, maximum specific activity was observed at *p*H 8.0 to 8.1 when the activation and reaction with substrate were performed at the same *p*H. The specific activity decreased rapidly as the *p*H was lowered, with only 10% of the maximum activity at *p*H 7.0, whereas the activity at *p*H 8.7 was only 20% below that observed at the optimum *p*H of 8.0 to 8.1. By comparison with the above information, the phosphotransacetylase from *C. kluyveri* was equally active in 0.1 M Tris-hydrochloride, glycyl-glycine, or histidine buffers at *p*H 8.1, but was inhibited by diethyl-barbiturate, Tris-citrate, and pyrophosphate (17).

Activation of the enzyme by cations. Phosphotransacetylase activity in the *C. acidiurici* preparations was not demonstrable unless ferrous or manganous ion was present in the activation and reaction mixture as shown in Table 2. Manganous ion could be substituted for ferrous ion but it was only 75% as effective. No activity was observed in the presence of 2 mM Fe^{3+}, Zn^{2+}, Mg^{2+}, Co^{2+}, Ni^{2+}, Sn^{2+}, Cu^{2+}, Mo^{6+}, Na^+, K^+, or NH_4^+.

The enzymes from *E. coli* B, *E. coli* K-12, and *C. kluyveri* are stabilized by the presence of 0.2 M ammonium sulfate during dilution and various purification procedures (2, 7, 14, 17). The *C. kluyveri* enzyme also exhibits greater heat stability in the presence of 0.2 M ammonium sulfate, retaining 50% of its activity after 5 min at 50 C. To a lesser extent, Na^+, K^+, and Mg^{2+} also increase the stability of this enzyme at higher temperatures (2, 16, 17). In the presence of ammonium sulfate, the phosphotransacetylase from *C. acidiurici* is extremely stable to freezing and thawing, and dilution and heat treatment, but remains highly unstable during gel filtration, dialysis, and column chromatography.

Stabilization of the enzyme by Fe^{2+} and dithiothreitol (DTT). Phosphotransacetylase loses approximately 50 to 60% of its activity when dialyzed for 8 hr against 0.05 M Tris buffer. Table 3 depicts the relative activity recovered after dialysis under various conditions when compared with an undialyzed sample of enzyme. When EDTA was incorporated into the dialysis buffer, either at 1 mM or 0.01 mM concentrations, there was no beneficial effect on the enzyme activity recovered,

TABLE 2. *Activation by cations*[a]

Phosphotransacetylase source	Cation	Acetyl-*S*-pantetheine generated (μmoles)	Activity (%)
Clostridium acidiurici	Fe^{2+}	3.1	100
C. acidiurici	Mn^{2+}	2.3	74[b]
C. acidiurici	None	0.0	0
Peptococcus glycinophilus	None	2.4	100[c]

[a] The activation and reaction mixtures contained the standard pantetheine assay reagents, except 2 μmoles of the indicated cation was substituted for Fe^{2+}. The activation and reaction tubes contained 8.2 μg of protein from *C. acidiurici* and 3.0 mg of protein from *P. glycinophilus*, respectively.

[b] The activity obtained with Mn^{2+} was compared to that observed with Fe^{2+}.

[c] The 60 to 70% ammonium sulfate fraction from *P. glycinophilus* contains a phosphotransacetylase which lacks a cation requirement.

TABLE 3. *Conditions necessary to stabilize phosphotransacetylase during dialysis*[a]

Additions to dialysis buffer			Phosphotransacetylase activity in dialysate	
EDTA (mM)	DDT[b] (mM)	$FeSO_4$ (mM)	Micromoles/ 10 min	Percent[c]
1.00			3,350	46
0.01			3,270	45
0.01	2.0		385	5
0.01		2.0	2,400	33
0.01	2.0	2.0	7,150	94

[a] Dialysis of 5.0 ml of 80 to 100% ammonium sulfate fraction (30 mg of protein/ml) was carried out using 2 liters of Tris buffer with additions as shown. Dialysis was carried out at 4 C for 8 hr under a nitrogen atmosphere. Any precipitate after dialysis was removed by centrifugation at 500 × *g* for 5 min before assaying for phosphotransacetylase activity by the standard pantetheine assay. All buffers were boiled, quickly cooled, and flushed with nitrogen before addition of dithiothreitol and $FeSO_4$.

[b] Dithiothreitol.

[c] Compared with undialyzed sample kept at 4 C for 8 hr.

since in both cases approximately 55% of the activity was lost. Addition of 2 mM $FeSO_4$ resulted in a 95% loss of activity, whereas addition of 2 mM DTT resulted in a 65% loss. However, addition of 2 mM $FeSO_4$ plus 2 mM DTT resulted in recovery of virtually 100% of the activity.

The stabilization by Fe^{2+} and DTT is also applicable to gel filtration and ion-exchange chromatography of the enzyme since both can be performed with negligible loss of enzyme activity if the columns are equilibrated and eluted with buffers containing 2 mM $FeSO_4$ and DTT. The degree of purification of the enzyme obtained under these conditions cannot be accurately assessed, however, due to the interference of DTT and Fe^{2+} with protein determinations.

Inhibition by orthophosphate and arsenate. The inhibition of phosphotransacetylase by orthophosphate and arsenate is shown in Fig. 3. Orthophosphate decreased the rate of acetyl-CoA generation 50% at 12 mM and 65% at 20 mM. Arsenate gave comparable results with 10 mM producing 50% and 20 mM producing 80% inhibition. These data may indicate that the enzyme possesses a site at which both arsenate and orthophosphate can interact in a similar fashion.

Arsenolysis studies. Because of the unusual property previously reported (12) that the phosphotransacetylase from *C. acidiurici* is not subject to uncoupling by arsenate, and our observation in the present studies of this same phenomenon, we explored the possibility that two forms of the enzyme may exist. It seemed possible that one form which is subject to arsenolysis might be present in crude extracts, but selectively eliminated in the purification procedures designed to concentrate the nonarsenolyzable form of the enzyme. As shown in Table 4 (tubes 1, 2, 3, and 10), variable quantities of enzyme in the presence of arsenate and catalytic quantities of either pantetheine or CoA show no decrease in acetyl-PO_4 resulting from arsenolysis. Fe^{2+} was omitted from these tubes (as well as from tube 6) to permit the possible demonstration of some form of phosphotransacetylase which reacted in the more conventional arsenolysis reaction typified by the *C. kluyverix* or *E. coli* enzymes (2, 7, 14, 17), but which might be inhibited by Fe^{2+}. In these tubes there was no decrease in acetyl-PO_4. Tube 4 contained the standard phosphotransacetylase system employed in these studies and showed that acetylthioester was generated from acetyl-PO_4. The addition of arsenate to the standard phosphotransacetylase assay mixture (tube 5), even with increased enzyme, did not result in the loss of acetyl-PO_4 beyond the amount which could be accounted for as acetylthioester (as in tube 4). Tube 6 confirms that demonstrable enzyme activity requires the presence of Fe^{2+} (compare with tube 4). Any loss of acetyl-PO_4, when employing either

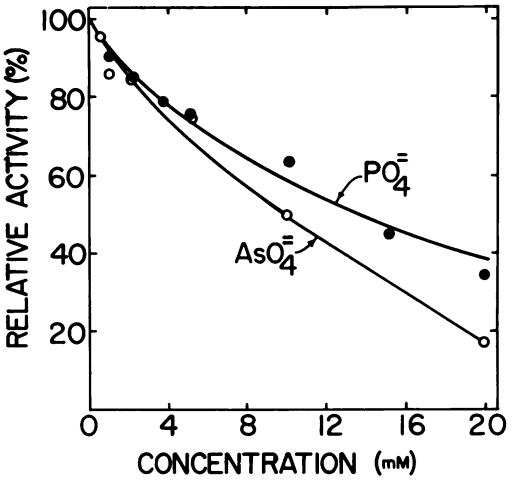

FIG. 3. *Inhibition of acetyl-S-CoA generation by orthophosphate and arsenate. The activation and reaction tubes contained the standard CoA assay reagents plus potassium arsenate or orthophosphate, pH 8.0, as indicated. The activation and reaction mixtures contained 0.25 μg of protein.*

pantetheine or CoA and excess enzyme, in the presence of arsenate (tubes 7A and 9A) could be accounted for as a comparable increase in acetylpantetheine or acetyl-CoA (tubes 7B and 9B), therefore demonstrating the ability of the enzyme in the crude extract to generate acetylthioester in the presence of arsenate.

The experiment shown in Table 5 was conducted to confirm the nonarsenolyzable nature of the purified enzyme from *C. acidiurici* and

TABLE 4. *Nonarsenolyzable phosphotransacetylase activity in crude extract from Clostridium acidiurici*

Tube no.	Added[a]						Recovered[b]	
	Fe^{2+}	AsO_4^{2-}	Acetyl acceptor		Crude extract (mg)	Ac-PO_4 (μmoles)	Ac-PO_4[c] (μmoles)	Acetyl-thioester (μmoles)
			PantSH (μmoles)	CoASH (μmoles)				
1		10	0.2		0.1	15	14.2	
2		10	0.2		0.5	15	16.0	
3		10	0.2		1.0	15	14.4	
4	2.0		10.0		0.1	15		1.8
5	2.0	10	10.0		0.5	15	14.4	
6			10.0		0.1	15		0.0
7A	2.0	10	10.0		1.0	15	13.6	
7B	2.0	10	10.0		1.0	15		1.2
8	2.0			10.0	0.1	15		0.6
9A	2.0	10		10.0	1.0	15	13.6	
9B	2.0	10		10.0	1.0	15		0.5
10		10		0.2	0.5	15	14.9	

[a] In addition to the indicated reagents added, the activation mixture contained Tris-hydrochloride, *p*H 8.0 (300 μmoles), in a total volume of 0.9 ml. After activation for 5 min at 37 C, the reaction was initiated by the addition of the acetyl-PO_4 (15 μmoles in 0.1 ml), and incubated an additional 10 min at 37 C.

[b] To measure acetyl-PO_4 remaining plus acetylthioester generated (total high-energy acetyl groups), the reaction was terminated by the addition of 1 ml of 1 mM *p*-hydroxymercuribenzoate, whereas reaction mixtures in which only acetylthioester was to be measured were terminated by the addition of 1 ml of 0.2 N HCl followed by boiling in a water bath for 6 min. Both total high-energy acetyl groups and acetylthioester were measured by the standard hydroxamate method.

[c] Acetyl-PO_4 recovered was determined from the difference of the concentration of total high-energy acetyl groups minus the acetylthioester recovered.

TABLE 5. *Comparison of phosphotransacetylases in the presence of arsenate*[a]

Enzyme source	Added			Recovered		
	Protein (μg)	Acetyl-PO_4 (μmoles)	AsO_4^{2-} (μmoles)	(I) Total acetyl units (μmoles)	(II) Acetyl-*S*-pantetheine (μmoles)	(III) Acetyl-PO_4[b] (μmoles)
Clostridium acidiurici	33.0	10	40	10.0	2.9	7.6
	3.3	10		10.2	9.5	0.7
Peptococcus glycinophilus	3,000.0	10	40	0.0	0.0	0.0
	3,000.0	10		10.3	2.1	8.2
None	0.0	10	40	10.0	0.0	10.0
	0.0	10		10.0	0.0	10.0

[a] The activation mixture contained (micromoles): Tris-hydrochloride, pH 8.0, 600; $FeSO_4$, 4.0; pantetheine, 40.0; KH_2AsO_4, *p*H 8.0, 80.0 (where indicated); and protein (as shown) in a total volume of 1.8 ml. Initiation of the reaction after activation for 5 min was carried out by the addition of 20 μmoles of acetyl-PO_4 (0.2 ml). One milliliter of the reaction mixture was immediately distributed into each of two tubes labeled I and II. The reaction in tube I was terminated after 10 min by the addition of 1 ml of 1 mM PHMB in 0.05 M phosphate, *p*H 5.5, for measurement of total acetyl units (I). The reaction in tube II was terminated after 10 min by the addition of 1 ml of 0.2 N HCl, and then boiled in a water bath for 6 min to selectively destroy the acetyl-PO_4 while preserving the acetyl-*S*-pantetheine (II).

[b] Acetyl-PO_4 (III) was calculated as the difference between (I) and (II).

compare these results with an ammonium sulfate fraction from *Peptococcus glycinophilus* which contains an arsenolyzable phosphotransacetylase (8). It can be seen that, although the generation of acetylthioester by the *C. acidiurici* enzyme is inhibited by arsenate, this activity can be demonstrated if high levels of enzyme are employed. The enzyme from *P. glycinophilus* is able to generate acetylthioester in the absence of arsenate, but differs from the *C. acidiurici* enzyme in that acetylthioester is not permitted to accumulate in the presence of arsenate, although acetyl-PO_4 is rapidly decomposed. In the absence of enzyme no acetylthioester was generated nor was arsenolysis observed.

Figure 4 demonstrates the ability of the *C. acidiurici* phosphotransacetylase to generate acetyl-CoA in the presence of arsenate without loss of total high-energy acetyl units. As the concentration of CoA increased, a decrease in acetyl-PO_4 was observed which was accompanied by a proportionate increase in acetyl-CoA. This phenomenon was observed both in the presence and absence of arsenate, although the reaction rate was decreased when arsenate was present. As the CoA concentration increased, the total acetyl units remained unchanged, both in the presence and absence of arsenate.

$H^{32}PO_4^{2-}$ exchange reaction. The phosphotransacetylase from *C. acidiurici* is capable of exchanging $H^{32}PO_4^{2-}$ with acetyl-PO_4 both in the presence and absence of arsenate (Table 6), although 10 mM arsenate decreased the exchange rate approximately fourfold. It was necessary to keep the combined arsenate and orthophosphate concentrations below 20 mM, since higher concentrations of either decreased the exchange reaction below demonstrable levels. The specific activity of the total phosphate (20 μmoles of acetyl-PO_4 plus 10 μmoles of ^{32}P-orthophosphate) present could be only one-third of that added as orthophosphate (1.9×10^5 dpm per 10 μmoles). Because of this fact, the maximum theoretical exchange between ^{32}P-orthophosphate and acetyl-PO_4 would yield acetyl-$^{32}PO_4$ with a specific activity of 6.3×10^4 dpm per μmole. The percent exchange observed in the absence of arsenate was 83% of this theoretical value, whereas in the presence of arsenate the level of exchange was decreased to 43% of this value.

Although orthophosphate acyltransferases with specificities for formyl-, acetyl-, propionyl-, butyryl-, isobutyryl-, or valeryl-phosphates, or both, have been partially purified from microbial sources (14, 15, 16, 22, 23), all appear closely related, since they undergo arsenolysis, possess similar *p*H optima, and re-

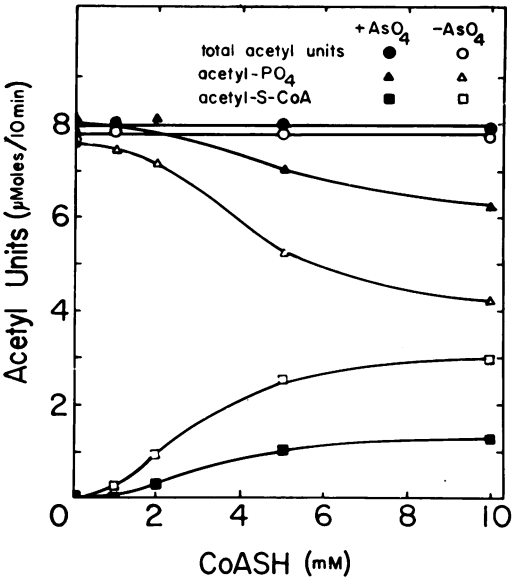

FIG. 4. *Generation of thioester in the presence of arsenate. The activation tubes contained (micromoles): Tris-hydrochloride (pH 8.0), 600; $FeSO_4$, 4.0; KH_2AsO_4, 20 (where indicated); CoASH, as indicated; and protein, 0.62 μg in 1.8 ml. The reaction was initiated by addition of 20 μmoles acetyl-PO_4 in 0.2 ml to the activation tubes. The reaction was terminated after 10 min of incubation by distributing 1 ml of the reaction mixture into 1 ml of 0.2 N HCl, to measure acetyl-S-CoA; and the remaining 1 ml into 1 ml of 0.1 M phosphate buffer (pH 5.5) containing 1 mM PHMB, to measure total acetyl units. Acetyl-S-CoA and total acetyl units were measured by the hydroxamate method.*

TABLE 6. *^{32}P Exchange in the presence of arsenate*[a]

Units of enzyme	$KHAsO_4^-$ added (μmoles)	$KH^{32}PO_4^-$ added (dpm)	Acetyl-$^{32}PO_4$[b] recovered (dpm)
750	10	190,000	27,000
375		190,000	52,000
0	10	190,000	0
0		190,000	0

[a] The activation tubes contained (in micromoles) the following in a total of 0.9 ml: Tris-hydrochloride, *p*H 8.0, 300; $FeSO_4$, 4.0; coenzyme A, 0.2; $KH^{32}PO_4^-$, *p*H 8.0 (specific activity 1.9×10^5 dpm per 10 μmoles), 10.0; 1 $KHAsO_4^-$, *p*H 8.0, 10.0 (where indicated); and enzyme as shown. The reaction was initiated by the addition of 20.0 μmoles acetyl-PO_4 in 0.1 ml and allowed to proceed for 20 min before being terminated by rapid cooling in an ice bath.

quire ammonium sulfate for stability during dialysis or dilution. The only representatives of this group which are stable to dialysis in the absence of ammonium sulfate are the phosphotransacetylase and phosphotransbutyrylase from *C. butyricum* (23).

Contrary to all other bacterial systems described, the purified phosphotransacetylase from *C. acidiurici* is nonarsenolyzable. The observation that this enzyme is able to generate acetylthioester and exchange $H^{32}PO_4^{2-}$ with acetyl-PO_4 in the presence of arsenate indicates that the catalytic site is capable of discriminating between orthophosphate and arsenate. Thus the ability of phosphate and arsenate to inhibit the enzyme activity to an approximately equal extent cannot be explained by interaction of these anions at the catalytic site, since arsenate appears to be excluded from this site. However, the data cited above suggest the possibility that there exists a noncatalytic site on the enzyme which is incapable of discriminating between phosphate and arsenate, and at which these ions could interact as negative homotropic effectors with an equal influence on the overall reaction kinetics (11).

The data presented here strengthen the original proposal by Sagers, Benziman, and Klein (12) that the ferrous ion requirement conveys the nonarsenolyzable nature on the enzyme, and point out many additional properties of this enzyme which merit further investigation. The enzyme can now be obtained in a highly purified state, and conditions necessary for the stabilization of the molecule are known. Several additional properties such as the very high catalytic constant and kinetic data will be reported in a subsequent publication.

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