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Properties of Acetyl-CoA Synthetase from *Pseudomonas fluorescens*

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Abstract: In *Pseudomonas fluorescens* grown on malonate as sole carbon source, acetyl-CoA synthetase was induced, suggesting that malonate is metabolized through acetate and then acetyl-CoA. Acetyl-CoA synthetase was purified 18.6-fold in 4 steps to apparent homogeneity. The native molecular mass of the enzyme estimated by a native acrylamide gel electrophoresis was 130 kDa. The enzyme was composed of two identical subunits with a molecular mass of 67 kDa. Optimum pH was 7.0. The acetyl-CoA synthetase showed typical Michaelis-Menten kinetics for the substrates, acetate, ATP and CoA, whose K_m values were calculated to be 33.4, 74.8, and 40.7 mM respectively. Propionate, butyrate and pentanoate were also used as substrates by the enzyme, but the rate of the formation of the CoA derivatives was decreased in the order of the increase in carbon number. The enzyme was inhibited by the group-specific reagents diethylpyrocarbonate, 2,3-butanedione, pyridoxal-5'-phosphate and N-bromosuccinimide. In the presence of substrates the inactivation rate of the enzyme, by all of the group-specific reagents mentioned above decreased, indicating the presence of catalytically essential histidine, arginine, lysine and tryptophan residues at or near the active site. Preincubation of the enzyme with ATP, Mg^{2+} resulted in the increase of its susceptibility to diethylpyrocarbonate, suggesting that ATP, Mg^{2+} may induce a conformational change in the active site exposing the essential histidine residue to diethylpyrocarbonate. The enzyme was acetylated in the presence of acetyl-CoA, indicating that this is one of acyl-enzyme.

Key words: acetyl-AMP, acetyl-CoA synthetase, *Pseudomonas fluorescens*.

Acetyl-CoA synthetase activity was detected during the course of an investigation of enzymes involved in the metabolism of malonate as sole carbon source in *Pseudomonas fluorescens*. The enzyme in *Pseudomonas fluorescens* is believed to catalyze the formation of acetyl-CoA directly from acetate and CoA in the presence of ATP. Acetate is generated from the decarboxylation of malonate by malonate decarboxylase located in the periplasmic space of the Pseudomonas (Kim and Byun, 1994). The activation of acetate to acetyl-CoA in bacteria is generally known *via* the sequential reaction of acetate kinase and phosphate transacetylase (Stadtman, 1952; Rose, 1955; Brown *et al.*, 1977), whereas the in mammalian (Chou and Lipmann, 1952; Jones and Lipmann, 1955; Webster, 1965; Dixon and Webb, 1979), plant (Hiatt and Evans, 1960; Huang and Stumpf, 1970) and fungi (Berg, 1956; Patel and Walt, 1987; Martinez-Blanco *et al.*, 1992) are by acetyl-CoA synthetase catalysis. Bacterial acetyl CoA synthetase have been however reported in *Bradyrhizobium japonicum* bacteroids (Preston *et al.*, 1990), *Acetobacter* aceti (O'Sullivan and Ettinger, 1976), *Rodospirillum rubrum* (Eisenberg, 1955), and *E. coli* (Brown *et al.*, 1977). Recently the importance of this enzyme as an acetyl donor to CheY protein in the chemotaxis has been also reported (Barak, 1992). In the reaction acetyl-AMP:enzyme complex is known to provide acetyl-group to CheY. Although acetyl-CoA synthetase in yeast is known to form a complex with acetyl-AMP, the acetyl-enzyme was also proposed to mediate the total ligase reaction (Wolfe *et al.*, 1988). By common testimony, acetyl-AMP:enzyme noncovalent complex is an intermediate of acyl-CoA ligase reactions. Therefore it is of interest to know the formation of acetyl-enzyme for the elucidation of their catalytic mechanism.

In this paper we present the isolation and the characterization of an acetyl-CoA synthetase from *P. fluorescens*. We also propose a catalytic mechanism from their inactivation and evidences for the formation of acetyl-AMP and acetyl-enzyme.

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Materials and Methods

Materials

Pseudomonas fluorescens ATCC 11250 was obtained from American Type Culture Collection. ATP, CoA, sodium acetate, sodium propionate, n-butyric acid, sodium tetraborate, ovalbumin, 2-mercaptoethanol, 1-anilinonaphthalene-8-sulfonate (ANS), diethylpyrocarbonate (DEP), N-bromosuccinimide (NBS) and pyridoxal-5′-phosphate (PLP) were purchased from Sigma Chemical Co. (St. Louis, USA). 2,3-butanedione was from Aldrich. Sephacryl S-300 and DEAE-Sephacel were obtained from Pharmacia Fine Chemicals. All other reagents were obtained commercially and were of analytical grade.

Culture and growth condition

P. fluorescens ATCC 11250 was grown on a medium containing (wt %) malonic acid, 1; NH_4Cl, 0.1; KH_2PO_4, 0.4; $MgSO_4 \cdot 7H_2O$, 0.03; $FeSO_4 \cdot 7H_2O$, 0.001, at 30℃ for 20 h with vigorous shaking. Cells were harvested in the late logarithmic phase of growth.

Enzyme assay

Acetyl-CoA synthetase activity was measured by the hydroxylamine assay method (Lipmann and Tuttle, 1945). This method was used for routine measurements during enzyme purification. The reaction mixture contained (in μmol); potassium phosphate, 50; sodium acetate, 10; $MgCl_2$, 10; ATP, 5; CoA, 0.1; neutral NH_2OH, 100; and enzyme and water in a total volume of 500 μl. After 30 min incubation at 37℃, the reaction was stopped by the addition of 250 μl of 10% TCA, followed by addition of 500 μl of $FeCl_3$ containing 0.66 N HCl. The precipitate was discarded after centrifugation and absorbance at 540 nm was measured. Molar extinction coefficient of acetohydroxamate under these conditions was 640 $M^{-1}cm^{-1}$.

Acetyl-CoA synthetase activity was also measured by a method based on the direct measurement of thioester formation at 232 nm. This assay method was used for characterization of the enzyme and in experiments involving the chemical modifications. The reaction mixture for this assay contained (in μmol) potassium phosphate buffer, pH 7.0, 100; sodium acetate, 0.2; $MgCl_2$, 1; ATP, 0.2; CoA, 0.1; and enzyme and water in a total volume of 1.0 ml. The mixture was incubated at 30℃ and the rates of increase in absorbance at 232 nm was simultaneously recorded by a Shimadzu UV 260 spectrophotometer (ε=4,500 $M^{-1}cm^{-1}$). Protein concentration was determined by the method of Bradford, using bovine serum albumin as a standard (Bradford, 1976).

Purification

The enzyme was purified from *P. fluorescens* grown on malonate as sole source of carbon and stored in a freezer (−70℃). Unless indicated otherwise, all steps in the procedure were carried out at 4℃. The cell paste (20 g) was resuspended in 0.1 M potassium phosphate buffer (pH 6.8) at a ratio of 1 g wet weight of cells to 0.5 ml of buffer. The cells were disrupted by French press. The disrupted cell suspension was resuspended in 0.1 M potassium phosphate buffer (pH 6.8) at a ratio of 1 g wet weight of cells to 6 ml of buffer. The suspension was then centrifuged at 20,000 ×*g* for 30 min and the pellet was discarded. To this crude extract, ammonium sulfate powder was added until concentration reached 30% saturation. The precipitate was centrifuged out. Ammonium sulfate was slowly added again to the supernatant to 45% saturation and the protein precipitate was collected after centrifugation at 20,000×*g* for 30 min. The pellet was resuspended in 5 ml of 0.1 M potassium phosphate buffer (pH 6.8) and was dialyzed against the same buffer. The enzyme solution was subjected to gel filtration on a Sephacryl S-300 column (2.8×90 cm) pre-equilibrated with 0.1 M potassium phosphate buffer (pH 6.8) and proteins were eluted in a descending manner with the same buffer. 4 ml fractions were collected every 8 min. The fractions containing acetyl-CoA synthetase were pooled and dialyzed overnight against 20 mM MOPS buffer (pH 7.2) containing 15% glycerol, and any insoluble material was removed by centrifugation. The enzyme solution was directly applied first to DEAE-Sephacel column (2.5×10 cm) equilibrated with 20 mM MOPS buffer (pH 7.2) containing 15% glycerol. After washing the column with the same buffer, the bound proteins were eluted with the same buffer using a KCl linear gradient (0~0.3 M) at a flow rate of about 3 ml/12 min. Acetyl-CoA synthetase was eluted from the column at about 0.15 M KCl concentration. The fractions containing acetyl-CoA synthetase were pooled and dialyzed overnight against 20 mM MOPS buffer (pH 7.2) containing 15% glycerol, and any insoluble material was removed by centrifugation. The enzyme solution was applied directly to second DEAE-Sephacel column (1.8×7 cm) equilibrated with 20 mM MOPS buffer (pH 7.2) containing 15% glycerol. The bound proteins were eluted with the same buffer using a KCl linear gradient (0~0.2 M) at a flow rate of about 2 ml/9 min. Acetyl-CoA synthetase was eluted from the column at about 0.15 M KCl concentration. The fractions containing acetyl-CoA synthetase were pooled and dialyzed overnight against 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol. Any

insoluble material was removed by centrifugation. The purified enzyme solution was stored at −20℃ and used for all subsequent experiment.

Inactivation

The reaction of the enzyme with DEP was initiated by adding appropriately diluted reagent to the enzyme solution in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol. The reaction was terminated by adding imidazole (pH 7.5) to make a 200 mM final concentration. The extent of inactivation was determined by measuring the residual enzyme activity at 30℃ on an aliquot removed from the reaction mixture. In the protection experiment the enzyme was preincubated with substrate for 5 min at 30℃ prior to the addition of DEP. Since the concentration of the commercial DEP is variable because of hydrolysis, the concentration of the reagent was determined for each diluted sample by its reaction with imidazole. The concentration was calculated from the increase in absorbance at 240 nm due to the formation of N-carbethoxy imidazole, using a molar absorbance of 3000 $M^{-1}cm^{-1}$ (Melchior and Fahrney, 1970).

The inactivation of acetyl-CoA synthetase by 2,3-butanedione was performed at 30℃ in the dark by the addition of the reagent into the enzyme solution in 20 mM sodium borate buffer (pH 8.0) containing 15% glycerol. Solutions of 2,3-butanedione were freshly prepared just before used by dissolving in distilled water. Aliquots were taken and the remaining enzyme activity in them were determined. In the protection experiment the enzyme was preincubated with substrate for 5 min at 30℃.

The inactivation of the enzyme by PLP was carried out at 30℃ in the dark by the addition of the reagent to the enzyme in 0.1 M potassium phosphate (pH 7.0) containing 15% glycerol. Since the formation of Shiff base by PLP was reversible, the aliquots taken from the reaction mixture were immediately treated with NaBH4 and the remaining activity in the aliquots were monitored. $NaBH_4$ (final conc. 30 mM) did not cause any effect on the enzyme activity. In the protection experiment the enzyme was preincubated with substrate for 5 min at 30℃.

The inactivation of the enzyme by NBS was performed at 30℃ for 5 min by the addition of the reagent to the enzyme in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol. The enzyme was preincubated with substrate for 5 min at 30℃ for the protection experiment. Oxidation of tryptophan residues by NBS was carried out in two cuvettes, one contained acetyl-CoA synthetase (1.5 μM) in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol and the other only the buffer. Successively, 1 μl aliquots of NBS (1.5 μM) were added to the sample as well as to the reference and absorbance at 280 nm was measured at 30℃ with a Shimadzu UV 260 spectrophotometer. The number of tryptophan residues (m) oxidized per mole of enzyme was calculated from the following equation (Spande and Witkop, 1967), $m=(1.31\times\Delta A_{280})/(5{,}500\times$ molarity of enzyme) where ΔA_{280} is the decrease in absorbance at 280 nm, 1.31 is an empirical factor and 5,500 is the molar extinction coefficient for the tryptophan at 280 nm.

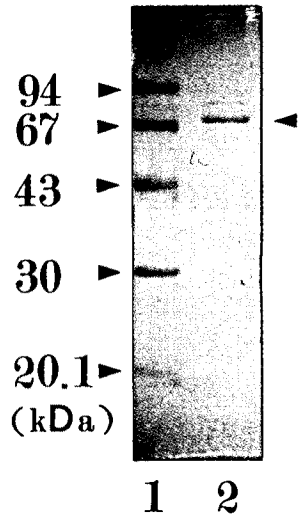

Fig. 1. SDS-PAGE of purified acetyl-CoA synthetase. Lane 1 : molecular weight standards (kDa); lane 2 : purified acetyl-CoA synthetase (2 μg). The enzyme was stained with Coomassie Brilliant Blue R-250.

Measurements of fluorescence intensity

The chemical modification of the enzyme by NBS and the interaction of the fluorescent dye ANS with the enzyme was analysed through the change in fluorescence intensity excited at 280 nm and 378 nm respectively, using a spectrofluorometer (Kontron, SFM25) with a quartz cuvette (light path, 1 cm) at pH 7.0 and 25℃.

Identification of acetyl-AMP

Acetyl-AMP was identified by autoradiogram of TLC analysis of reaction products. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.1, 5 μM $MgCl_2$, 1 mM acetate, [α-^{32}P]ATP (0.2 pmol), and the enzyme (6 pmol) in a total volume of 25 μl. After incubation aliquots of 1 μl were spotted onto a PEI-cellulose plate at the indicated time. When CoA was added, the final concentration was maintained to 0.5 mM.

Table 1. Purification of acetyl-CoA synthetase from *Pseudomonas fluorescens* grown on malonate

Purification	Total proteins (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Fold
Crude	2655	119.0	0.045	100	1
Ammonium sulfate precipitation	375	72.5	0.193	60.9	4.3
Gel filtration	209	48.3	0.231	40.6	5.2
1st DEAE-Sephacel	23.1	12.1	0.524	10.2	11.7
2nd DEAE-Sephacel	9.6	8.0	0.833	6.7	18.6

Identification of acetyl-enzyme

The enzyme (0.5 nmol) was incubated with [2-^{14}C] acetyl-CoA (1 nmol) in 0.1 M sodium phosphate buffer, pH 7.1, in a total volume of 60 μl. The reaction mixture was loaded on SDS PAGE and radioactivity on the dried gel was monitored by a phosphorimager.

Results

Isolation

Acetyl-CoA synthetase induced in malonate-grown *P. fluorescens* was purified by the method described above in an electrophoretically homogeneous form (Fig. 1). In any case the combination of procedures summarized in Table 1 resulted in over about 19 fold purification of the enzyme with about 7% overall yield.

General properties

The M_r of the enzyme was estimated to be 130,000 by a pore gradient gel electrophoresis. SDS PAGE of the enzyme revealed only one protein band with a M_r of 67,000, indicating that it is a homodimeric enzyme composed of two polypeptide (Fig. 1). The enzyme showed the highest activity with acetate under the condition, pH 7.0, temperature 30℃, and it requires metal ion, Mg^{2+} or Mn^{2+}. With increasing concentration of substrate, the rate of acetyl-CoA formation was increased and Michaelis-Menten type substrate saturation pattern was obtained. K_m and V_{max} values were calculated to be 33.4 μM and 1.57 μM/min for acetate, 74.8 μM and 2.03 μM/min for ATP, and 40.7 μM and 1.68 μM/min for CoA, respectively. The enzyme also used acetic, propionic, butyric and valeric acids as substrates but with the different degree of reactivity (100, 80.1, 10.2, and 5.2%, respectively). The purified enzyme could be stored at −20℃ without any loss of activity for many months (Table 2).

Table 2. Properties of acetyl-CoA synthetase from *Pseudomonas fluorescens* grown on malonate

Molecular size	137,000 dalton	
Subunit	67,000 dalton identical	
Optimum pH	7.0	
Metal iion requirement	Mg^{2+} or Mn^{2+}	
Kinetic constants	K_m (μM)	V_{max} (μM/min)
for acetate	33.4	1.57
for ATP	74.8	2.03
for CoA	40.7	1.68
Substrate specificity	(% based on rate)	
acetate	100	
propionate	80.1	
butyrate	10.2	
valerate	5.2	
Stability	stable at −20℃	

Inactivation

DEP irreversibly inactivated acetyl-CoA synthetase and the inactivation was dependent on DEP concentration and incubation time (Fig. 2, A). Under the applied temperature, pH, concentration and composition of the buffer, the pseudo-first-order rate constant for hydrolysis of DEP itself (k') was determined to be 0.101 min^{-1} in this system. Considering the self hydrolysis of DEP. the fraction of enzyme molecules retaining full activity (A/A_o) was expressed as:

$$\ln(A/A_o) = -(k/k')I(1-e^{-k't})$$

where I is the initial concentration of DEP; k is the bimolecular rate constant for reaction of enzyme with the reagent; t is time (min) for incubation with DEP; and k' is the pseudo-first-order rat constant for hydrolysis of the reagent. With the k' value, the data obtained from experiments on the inactivation of the enzyme for several concentrations of DEP (0.2, 0.4, 0.6, 0.8, 1.0 mM) gave straight lines in a plot of ln (A/A_o) versus $(1-e^{-k't})/k'$. The inactivation followed pseudo-first-order kinetics, and a plot of kobs versus DEP concentration was linear, indicating that the modification is the result of a simple bimolecular reaction between DEP and an enzyme residue. The second-order rate constant of DEP modification was 795 M^{-1}min^{-1}. A double logarithmic plot of the reciprocal of the half-time of inac-

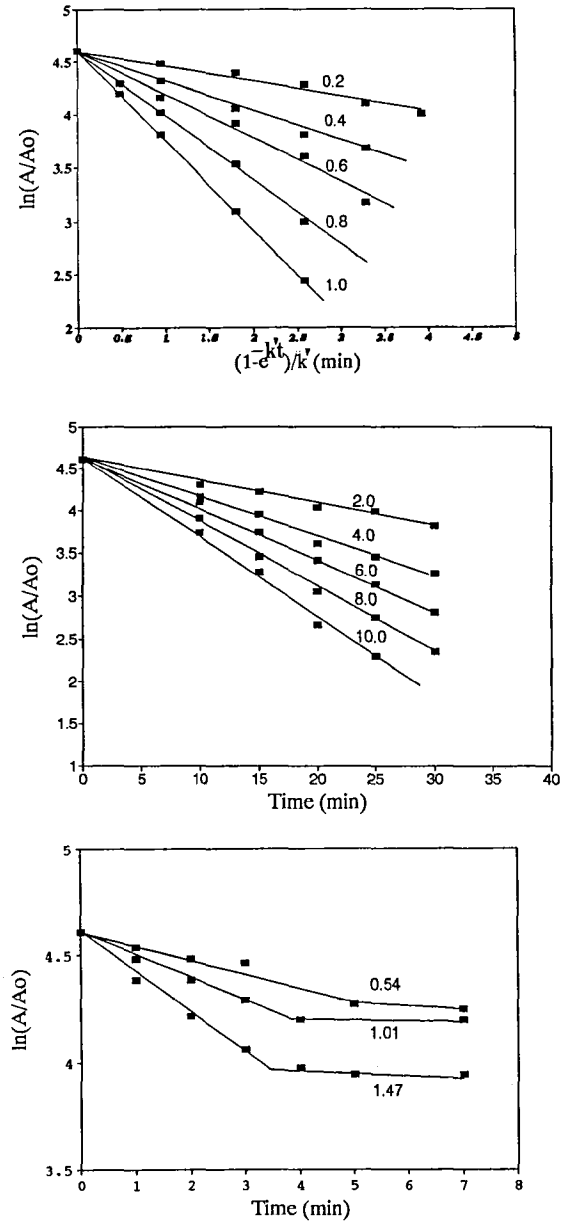

Fig. 2. Inactivation of acetyl-CoA synthetase by DEP, 2,3-butanedione and PLP. (A) The enzyme (2.7 μM) was incubated with different concentrations of DEP in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol, at 30℃. The numbers on the slopes indicated DEP concentration in mM. (B) The enzyme (2.3 μM) was incubated with different concentrations of 2,3-butanedione in 20 mM sodium borate buffer (pH 8.0) containing 15% glycerol, at 30℃. The numbers on the slopes indicated 2,3-butanedione concentration in mM. (C) The enzyme (3.4 μM) was incubated with different concentrations of PLP in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol, at 30℃. The numbers on the slopes indicated PLP concentration in mM.

tivation against DEP concentration yields a reaction order of 1.09 with respect to the DEP, indicating that one molecule of DEP react with one molecule of enzyme composed of two identical subunits (Table 3).

The inactivation of the enzyme by DEP was protect-

Table 3. Inactivation of acetyl-CoA synthetase from *Pseudomonas fluorescens* grown on malonate

Inactivation	k_2 ($M^{-1}min^{-1}$)	n	Essential amino acid	Protection
DEP	795	1.09	His	Acetate
2,3-Butanedione	9.2	0.8	Arg	ATP
PLP	50.2	1.07	Lys	Acetate
NBS		2[a]	Trp	CoA

[a]Number of residues modified by NBS for the complete inactivation.

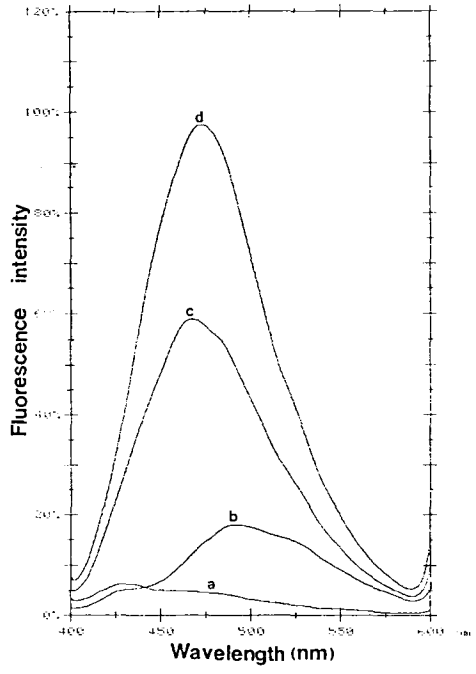

Fig. 3. Fluorescence emission spectra of ANS bound to acetyl-CoA synthetase. The excitation wavelength was 378 nm. The mixture contained (a) 5 μM enzyme in 0.1 M potassium phosphate (pH 7.0) containing 15% glycerol, (b) 10 μM ANS, (c) 5 μM enzyme and 10 μM ANS and (d) 5 μM enzyme, 2 mM ATP/Mg^{2+} and 10 μM ANS. In the case of (d), the enzyme was preincubated with ATP/Mg^{2+} for 5 min at 30℃ before the addition of ANS. ATP had no effect on the fluorescence emission of ANS in the absence of enzyme.

ed by the preincubation of the enzyme with either acetate or propionate. This indicates that the DEP modified residue is located at or near the acetate binding region. On the other hand, the preincubation of the enzyme with ATP/Mg^{21} resulted the increase in inactivation. It suggest that ATP/Mg^{2+} binding to the enzyme may induce conformational change of the enzyme active site, and provides susceptible position of the essential histidine residue to DEP. Conformational change of the enzyme by binding of ATP/Mg^{2+} was determined by measuring the ANS binding capacity. The addition

of ANS to acetyl-CoA synthetase in 0.1 M potassium phosphate (pH 7.0) containing 15% glycerol resulted in a large increase in fluorescence emission (Fig. 3). The emission peak appeared at 465 nm. ANS or enzyme alone gave negligible emission under these conditions. The fluorescence intensity of the enzyme was markedly increased by ATP/Mg^{2+} with no significant change in the spectral position, suggesting that ATP/Mg^{2+} produced a pronounced alteration in the enzyme conformation.

2,3-Butanedione is considered to be highly selective for modification of arginine residues in borate buffer (Lundblad and Noyes, 1988). Treatment of acetyl-CoA synthetase with 2,3-butanedione in 20 mM sodium borate buffer (pH 8.0) containing 15% glycerol resulted in a time-dependent loss of enzyme activity(Fig. 2, B). Plots of the logarithm of remaining activity versus time at different concentrations of 2,3-butanedione indicated pseudo-first-order kinetics. The straight line of the plot of pseudo-first-order rate constants versus concentrations of 2,3-butanedione indicates that the modification was simply bimolecular (data not shown). The second-order rate constant for modification was 9.2 $M^{-1}min^{-1}$. A double logarithmic plot of the reciprocal of the half-time of inactivation against 2,3-butanedione concentration yields a reaction order of 0.8 with respect to 2,3-butanedione (Table 3), indicating that approximately 1 molecule of 2,3-butanedione reacts with 1 molecule of enzyme. ATP/Mg^{2+} protected the enzyme from inactivation by 2,3-butanedione, indicating that one arginine residue essential for acetyl-CoA synthetase activity is located at or near the ATP binding region.

Treatment of acetyl-CoA synthetase with varying concentrations of PLP at pH 7.0 resulted in time dependent loss of enzyme activity. The inactivation of the enzyme was biphasic (Fig. 2C). During the initial phase a rapid loss of the enzyme activity was observed and subsequently followed by a slower time-dependent loss of the enzyme activity. The inactivation followed pseudo-first-order kinetics and a plot of kobs versus PLP concentration was linear (data not shown), indicating that the modification is the result of a simple bimolecular reaction between PLP and an enzyme residue. The second-order rate constant of PLP modification was 50.2 $M^{-1}min^{-1}$. A double logarithmic plot of the reciprocal of the half-time of inactivation against PLP concentration yields a reaction order of 1.07 with respect to PLP (Table 3), indicating that inactivation results by the reaction of approximately 1 mol of PLP with 1 mol of enzyme. The inactivation of the enzyme by PLP was protected by the addition of either acetate or propionate (Table 3). This indicates that the PLP modified residue is located at or near the acetate binding region.

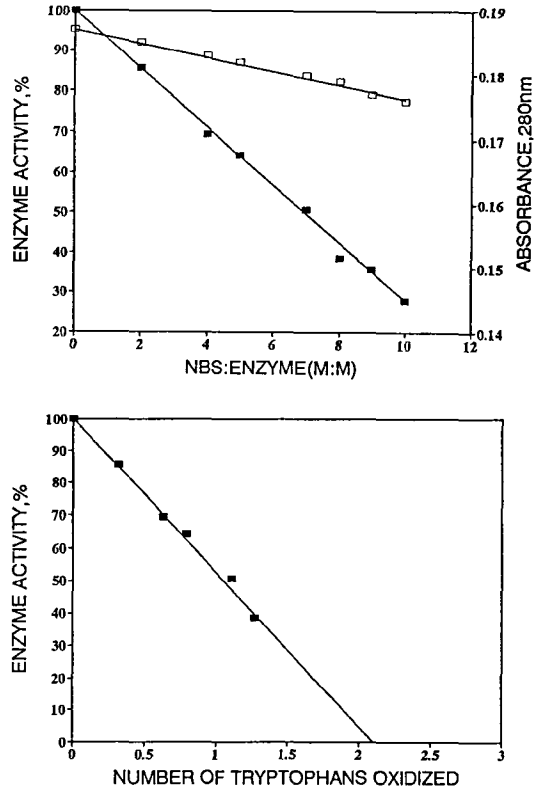

Fig. 4. (A) Activity and UV absorbance changes of acetyl-CoA synthetase as a function of the molar excess of NBS. Purified enzyme (1.5 μM) was incubated with different concentration of the reagent in 0.1 M potassium phosphate buffer (pH 7.0) containing 15% glycerol at 30℃. Enzyme activity is expressed as percent of an untreated control. (B) Quantitative relationship between oxidized tryptophan residues and acetyl-CoA synthetase activity. The amount of tryptophan oxidized was estimated from the decrease in absorbance at 280 nm according to the method of Spande and Witkop (1967).

NBS inactivated very rapidly acetyl-CoA synthetase with concomitant decrease in absorbance at 280 nm and the amount of inactivation varied with the concentration of NBS (Fig. 4, A). The stoichiometry of the inactivation of an enzyme with NBS can be calculated by the determination of the loss of enzyme activity and the spectral change at 280 nm (Spande and Witkop, 1967). The complete inactivation of acetyl-CoA synthetase with NBS yielded the oxidation of two tryptophan residues (Fig. 4, B). The chemical modification of the enzyme by NBS was also observed through the change in the fluorescence intensity of the enzyme excited at 280 nm (Fig. 5). When excited at 280 nm, the enzyme shows fluorescence emission spectra having a peak at about 329 nm. The fluorescence was quenched by modification of the enzyme with NBS. The degree of fluorescence quenching increased as the added NBS concentration was increased, as shown in Fig.

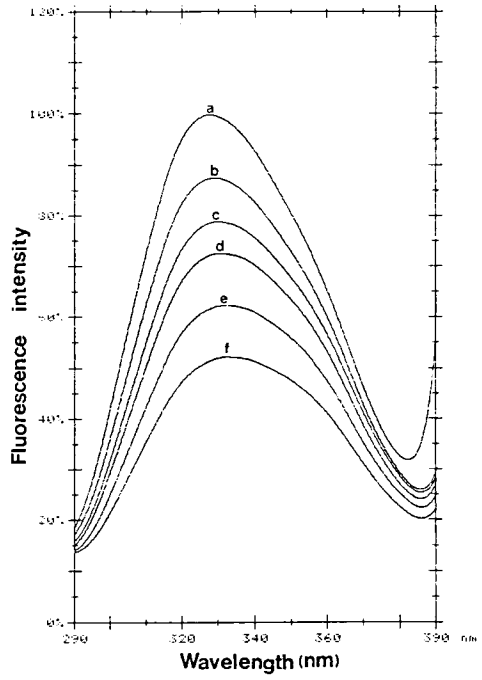

Fig. 5. Fluorescence emission spectra of acetyl-CoA synthetase as a function of molar excess of NBS. The excitation wavelength was 280 nm. [NBS] : [Enz] ratios are (a) 0.0, (b) 2.0, (c) 4.0, (d) 6.0, (e) 8.0, (f) 10.0. The spectra were recorded at five minutes after mixing the enzyme and NBS solutions. The enzyme concentration was 1.5 μM.

5. Inactivation by NBS was partially prevented by ATP/Mg^{2+}. Complete protection from inactivation, however, was afforded by preincubation of the enzyme with CoA before the NBS treatment (Table 3). This indicates that NBS modified residues are located at or near the CoA binding region.

Formation of acetyl-AMP

The formation of acetyl-AMP was confirmed by the autoradiogram of TLC analysis of a reaction mixture containing the enzyme, acetate, and [α-^{32}P]ATP, which revealed the presence of a novel nucleotide derivative that was neither AMP, ADP, or ATP (Fig. 6). The intensity of the spot gradually increased with incubation time. Also, the nucleotide disappeared gradually with the addition of CoA, concomitant with the production of AMP. This result clearly indicate that the novel nucleotide is acetyl-AMP.

Acetylation of the enzyme

When the enzyme was incubated with [2-^{14}C]acetyl-CoA, which is a product, it was found that the enzyme was labeled isotopically (Fig. 7). That is, the isotopically labeled enzyme was monitored by a phosphorimager on the SDS PAGE plate but only when the sample

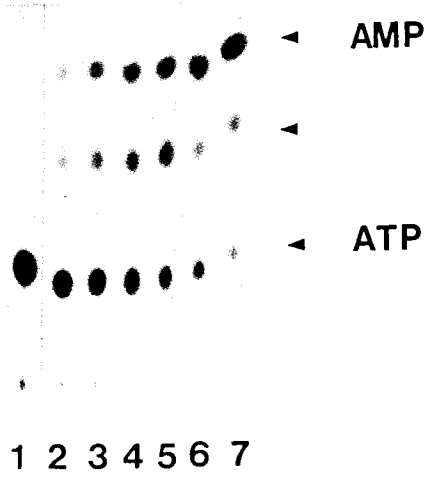

Fig. 6. Autoradiogram of TLC analysis of the partial reaction products using [α-^{32}P]ATP. The reaction mixture contained [α-^{32}P] ATP and acetate as substrates. After 4 min, aliquots of 10 μl were taken and mixed with CoA. The mixture containing CoA was separately incubated and aliquots of 1 μl were spotted on to a PEI-cellulose plate at the indicated time. Lane 1 : control (with boiled enzyme); lanes 2~5 : 1 min, 4 min, 8 min, 12 min from the reaction mixture in the absence of CoA respectively. lanes 6~7 : 4 min and 8 min after the addition of CoA respectively.

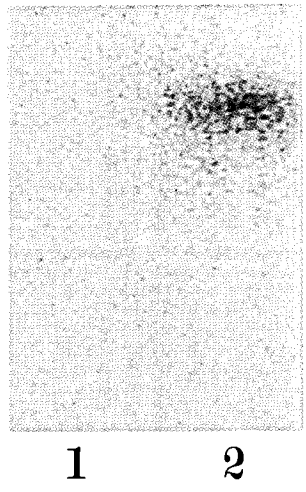

Fig. 7 Identification of [^{14}C]acetyl-enzyme using Phosphorimager. The enzyme was incubated with [2-^{14}C]acetyl-CoA during 10 min. Lane 1 : control (boiling after the reaction); lane 2 : reaction mixture. The reaction mixture was loaded on SDS-PAGE and radioactivity on the dried gel was monitored by a phosphorimager.

was not treated by heat. This result indicates clearly that the enzyme is acetylated by acetyl-CoA and the bond between the enzyme and acetyl group might be heat-and base-labile. Considering three essential amino acid residues on acetylation/ATP binding site, histidine might be the most probable candidate for the acetylation site.

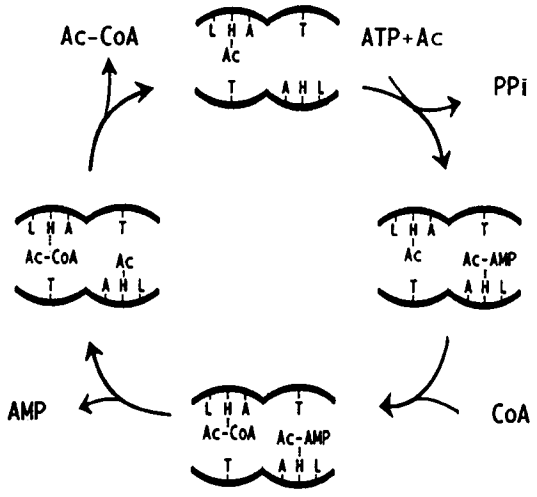

Fig. 8. Proposed catalytic model of acetyl-CoA synthetase from *Pseudomonas fluorescens*. A : arginine, Ac : acetate, Ac-AMP : acetyl-AMP, Ac-CoA : acetyl coenzyme A, Ac-H : acetyl-histidine, H : histidine, L : lysine, T : tyrosine.

Discussion

Bacterial acetyl-CoA synthetase has been known to be involved primarily in biosynthetic functions. The enzyme has been purified and characterized from a number of mammalian, plant tissues and fungi, but a few bacterial enzymes from *Acetobacter aceti*, *Rhodospirillum* and *Bradyrhizobium japonicum* bacteroids has been identified. In bacteria, the primary route of activation of acetate to acetyl-CoA is known to be via the sequential reactions of acetate kinase and phosphate transacetylase. However, acetyl-CoA synthetase induced in *P. fluorescens* grown on malonate is involved in the utilization of malonate. In this bacteria acetyl-CoA synthetase is believed to be involved in the activation of acetate generated by malonate decarboxylase from malonate. It was known that the key enzymes of glyoxylate cycle, isocitrate lyase (Jang and Kim, 1982) and malate synthase (Chae and Kim, 1987), and malonate decarboxylase (Kim and Byun, 1994) are induced in *P. fluorescens* grown on malonate.

The purification of acetyl-CoA synthetase resulted in only a 18.6-fold increase in the specific activity over the crude extract, indicating that *P. fluorescens* grown on malonate is a hyperproducer of acetyl-CoA synthetase. The K_m values for acetate, ATP and CoA were 33.4, 74.8 and 40.7μM respectively. These K_m values are much lower than those for acetyl-CoA synthetase from other sources.

Treatment of the enzyme with group-specific reagents DEP, 2,3-butanedione, PLP and NBS resulted in loss of enzyme activity. The analysis of kinetics of inactivation showed that the loss of enzyme activity was due to modification of a single histidine, arginine and lysine residue located at or near the active site. The relationship between activity loss and the number of tryptophan residues modified demonstrated that two tryptophan residues are at or near the active site. Acetate or propionate protected the enzyme from inactivation by either DEP or PLP. This mode of protection implicates an essential histidine and lysine residue in acetate binding by providing the positive charge to interact the negative charge of the carboxyl group of acetate. Preincubation of the enzyme with ATP/Mg^{2+} appeared to enhance the inactivation of the enzyme by DEP. The binding of ATP/Mg^{2+} induce a conformational change of the enzyme, which makes the essential histidine residue more accessible to modification by DEP. Conformational change of the enzyme by binding of ATP/Mg^{2+} was confirmed by the change of ANS binding capacity of the enzyme. ANS and their derivatives have been extensively used to study the conformations of proteins (Weber and Young, 1964; McClure and Edelaman, 1967; Aoe *et al.*, 1970).

The inactivation of acetyl-CoA synthetase by 2,3-butanedione was found to be buffer dependent (data not shown). Borate buffer specifically enhanced the rate of inactivation. The inactivation in potassium phosphate buffer and Tris-HCl was much slower. The inactivation of the enzyme by 2,3-butanedione was protected by the preincubation of the enzyme with ATP/Mg^{2+}. Arginine residue located at AMP binding site has been reported in glycogen phophorylase (Dreyfus, 1980). Malonyl-CoA synthetase from *Rhizobium trifolii* has a arginine residue which is located at or near the ATP binding region (Lee and Kim, 1993). It seems reasonable to state that a catalytically essential arginine residue is located at ATP binding site of acetyl-CoA synthetase.

The modification of tryptophan residues was monitored by the changes in the absorbance of acetyl-CoA synthetase at 280 nm and fluorescence intensity of the enzyme excited at 280 nm. When excited at 280 nm, the fluorescence intensity of the enzyme modified with NBS was quenched, indicating that NBS modified tryptophan residues of the enzyme. Protection against NBS-mediated inactivation exclusively by CoA clearly implicates the location of two essential tryptophan residues at or near the CoA binding site. Catalytically essential tryptophan residue located at methylmalonyl-CoA binding region has been reported in transcarboxylase from *Propionibacterium shermanii* (Kumar *et al.*, 1988). These residues may function to hold CoA and facilitate proper orientation for the substrate by means of hydrophobic interaction.

The determination of amino acid residues involved in substrate binding and catalysis is fundamental for

understanding of the catalytic mechanism. From the data, 1) an identically dimeric structure, 2) catalytically essential one histidine, and one lysine residue, respectively, at acetate binding site, 3) catalytically essential one arginine at ATP binding site, 4) increase in susceptibility for the modification of the enzyme by DEP in the presence of ATP, indicating a conformational change to the structure beneficial for acetylation, 5) catalytically essential two tryptophan residues at CoA binding site, 6) formation of acetyl-AMP, and 7) formation of acetyl-enzyme having chemical bond heat- and base-labile. we propose a catalytic mechanism of acetyl-CoA synthetase from *P. fluorescens* (Fig. 6). That is, each subunit has two separate domains, ATP binding/acetylation and CoA binding sites. One acylation site of the enzyme is acetylated, whereas other site is open for the next round of the catalysis. The histidine, arginine, and lysine located at the acetylated-active site might not be susceptible by DEP, 2,3-butanedione, and PLP, respectively. ATP react with acetate to form acetyl-AMP at the ATP binding/acetylation site, followed by the acetylation of the enzyme by acetyl-AMP. CoA bound on other subunit attack acetyl-enzyme as a nucleophile to form acetyl-CoA. It seems that the two pair of active sites may alternatively participate for the catalysis.

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References

Aoe, H., Samgadharan, M. G. and Pogell, B. M. (1970) *J. Biol. Chem.* **245**, 6383.

Barak, R., Welch, M., Yanovsky, A., Oosawa, K. and Eisenbach, M. (1992) *Biochemistry* **31**, 10099.

Berg, P. (1956) *J. Biol. Chem.* **222**, 991.

Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248.

Brown, T. D. K., Jones-Mortimer, M. C. and Konberg, H. L. (1977) *J. Gen. Microbiol.* **102**, 327.

Chae, H. Z. and Kim, Y. S. (1987) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.)* **20**, 239.

Chou, T. C. and Lipmann, F. (1952) *J. Biol. Chem.* **196**, 89.

Dixon, M. and Webb, E. C. (1979) Enzymes, 3rd ed., Academic Press, New York.

Dreyfus, M., Vandenbunder, B. and Buc, H. (1980) *Biochemistry* **19**, 3634.

Eisenberg, M. A. (1955) *Biochim. Biophys. Acta* **16**, 58.

Hiatt, A. J. and Evans, H. J. (1960) *Plant Physiol.* **35**, 673.

Huang, K. P. and Stumpf, P. K. (1970) *Arch. Biochem. Biophys.* **140**, 158.

Jang, S. H. and Kim, Y. S. (1982) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **15**, 177.

Jones, M. E. and Lipmann, F. (1955) *Methods Enzymol.* **1**, 585.

Kumar, G. K., Beegen, H. and Wood, H. G. (1988) *Biochemistry* **27**, 5972.

Lee, S. C. and Kim, Y. S. (1993) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **26**, 286.

Lipmann, F. and Tuttle, L. C. (1945) *J. Biol. Chem.* **159**, 21.

McClure, W. O. and Edelaman, G. M. (1967) *Biochemistry* **6**, 559.

Martinez-Blanco, H., Reglero, A., Fernandez-Valverde, M., Ferrero, M. A., Moreno, M. A., Penalva, M. A. and Luengo, J. M. (1992) *J. Biol. Chem.* **267**, 5474.

Melchior, W. B., Jr. and Fahmey, D. (1970) *Biochemistry* **9**, 2510.

Sullivan, J. and Ettinger, L. (1976) *Biochim. Biophys. Acta* **450**, 410.

Patel, S. S. and Walt, D. R. (1987) *J. Biol. Chem.* **262**, 7132.

Rose, I. A. (1955) *Methods Enzymol.* **1**, 591.

Preston, G. G. , Wall, J. D. and Emerich, D. W. (1990) *Biochem. J.* **267**, 179.

Spande, T. F. and Witkop, B. (1967) *Methods Enzymol.* **11**, 498.

Stadtman, E. R. (1952) *J. Biol. Chem.* **196**, 527.

Weber, G. and Young, L. B. (1964) *J. Biol. Chem.* **239**, 1415.

Webster, L. T. Jr. (1965) *J. Biol. Chem.* **240**, 4158.

Wolfe, A. J., Conley, M. P. and Berg, H. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 6711.