high affinity for phenol, $K_s(I) = 5 \times 10^{-7}$ to $5 \times 10^{-6}$ M, and may thus represent the binding site for effector. It is hard to decide whether the second dissociation constant, $K_s(II) = (1-8) \times 10^{-3}$ M, represents just another binding site of a much lower affinity or reflects a more indiscriminate binding of phenol to various regions on the enzyme. Either case may cause a collapse of a specific conformation required in the catalytic cycle, concomitant with return of the spectrum to that of the uncomplexed enzyme (Figure 7B). Both cases would correlate with the phenomenon of excess substrate inhibition observed with phenol hydroxylase (Neujahr & Kjellén, 1978, 1980).

Acknowledgments

I am indebted to Annette Elmblad and Ulla Hägglund for the many fluorometric determinations, only a part of which has been included in the present paper.

**Registry No.** FAD, 146-14-5; $CN^-$, 57-12-5; $N_3^-$, 14343-69-2; $F^-$, 16984-48-8; $SCN^-$, 302-04-5; $Cl^-$, 16887-00-6; $NO_3^-$, 14797-55-8; $I^-$, 20461-54-5; $Br^-$, 24959-67-9; $CH_3COO^-$, 71-50-1; $CCl_3COO^-$, 14357-05-2; Mg, 7439-95-4; ethylene glycol, 107-21-1; phenol, 108-95-2; phenol hydroxylase, 37256-84-1.

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# Effects of Nucleotides on a Cold Labile Acetyl-CoA Hydrolase from the Supernatant Fraction of Rat Liver†

Fumihide Isohashi, Yoko Nakanishi, and Yukiya Sakamoto*

ABSTRACT: An acetyl-CoA hydrolase that is labile at low temperature was purified to homogeneity from the supernatant fraction of rat liver. The monomeric molecule, estimated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, had a molecular weight of about 63 000, while that of the purified enzyme, estimated by gel filtration, was 135 000. Thus, the enzyme consists of two subunits of identical molecular weight. On addition of adenosine 5′-triphosphate (ATP) or adenosine 5′-diphosphate (ADP) at 25 °C, the dimeric form of the enzyme aggregated to tetrameric forms ($M_r$ 242 000 and $M_r$ 230 000, respectively), whereas addition of adenosine 5′-monophosphate had little effect on enzyme association ($M_r$ 145 000). When ATP was removed from the ATP-treated tetrameric enzyme by dialysis, the tetramer was mostly dissociated into the dimeric form. The apparent $K_m$ values for acetyl coenzyme A of the dimeric enzyme and tetrameric enzyme, reconstituted from the former in the presence of 2 mM ATP, were 170 μM and 60 μM, respectively. The purified dimeric enzyme was inactivated by exposure to lower temperature, especially below 10 °C. The various nucleotides tested partially stabilize the dimeric enzyme at low temperature, ATP being the most effective. Sucrose density gradient centrifugation showed that loss of catalytic activity by cold treatment was accompanied by dissociation of the dimer and tetramer into protomer.

Acetyl-CoA hydrolase (EC 3.1.2.1) catalyses the reaction acetyl-CoA + $H_2O \rightleftarrows$ CoA + acetate. This enzyme was first found in pig heart by Gergely et al. (1952) and has subsequently been found in many mammalian tissues (Hepp et al., 1966; Anderson & Erwin, 1971; Quraishi & Cook, 1972; Knowles et al., 1974; Robinson et al., 1976; Matsuda & Yoshida, 1976; Bernson, 1976; Matsuda et al., 1978; Snoswell & Tubbs, 1978; Grigat et al., 1979). In rats, the activity is high in the liver where enzyme is predominantly located in the mitochondria (Knowles et al., 1974; Snoswell & Tubbs, 1978; Grigat et al., 1979).

F. Isohashi and M. F. Utter (unpublished experiments; cf. Prass et al., 1980) first observed activity of ATP[1]-activated acetyl-CoA hydrolase in rat liver homogenates prepared in buffer containing 1.5 M sucrose.[2] This enzyme, which appears to have an extramitochondrial location, had presumably not been detected previously because of its extreme cold lability in the crude homogenate (Prass et al., 1977, 1980) and in excised liver (Isohashi et al., 1981). Its hydrolytic activity in the presence of ATP in rat liver was demonstrable only when liver homogenate was prepared in buffer containing 1.5 M

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[1] Abbreviations: AMP, ADP, and ATP, adenosine 5′-mono-, 5′-di-, and 5′-triphosphates; $NAD^+$ and NADH, the oxidized and reduced forms of the coenzyme nicotinamide adenine dinucleotide; $NADP^+$ and NADPH, the oxidized and reduced forms of the coenzyme nicotinamide adenine dinucleotide phosphate; $PP_i$, inorganic pyrophosphate; cAMP, adenosine cyclic 3′,5′-phosphate; ITP, UTP, CTP, GTP, and TTP, inosine, uridine, cytidine, guanosine, and thymidine 5′-triphosphates; PMSF, phenylmethanesulfonyl fluoride; DTT, DL-dithiothreitol; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate.

[2] Isohashi–Leiter–Utter medium (ILU medium) (Atkin et al., 1979; Murphy et al., 1981) consisting of 50 mM potassium phosphate buffer (pH 7.4), 1.5–2.0 M sucrose, and 0.5 mM EDTA.

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sucrose[2] at 4 °C or at 25 °C, or in isotonic buffer at room temperature (Prass et al., 1980). Prass et al. (1977, 1980) were the first to purify an enzyme of this class to homogeneity using ATP as enzyme stabilizer and an ATP–agarose column at room temperature and reported some properties of the enzyme: The molecular weight of the purified enzyme was about 240 000 (determined by gel filtration) or 340 000 (determined by equilibrium centrifugation), and the subunit molecular weight was 64 000, suggesting that the enzyme consists of at least four subunits. The purified enzyme was activated by ATP and completely inhibited by ADP. The presence of 2 mM ATP in the assay medium lowers the apparent $K_{0.5}$ for acetyl-CoA from 1.3 mM (no nucleotide) to 60 $\mu$M. This regulation by nucleotide was not dependent on the presence of $Mg^{2+}$, but a high $Mg^{2+}$ (10 mM) concentration inhibited ATP activation. ATP does not appear to be a substrate, and its effect is probably due to allosteric interaction. Addition of acetate, NADH, NAD, NADPH, NADP, AMP, $PP_i$, citrate, L-carnitine, acetyl-L-carnitine, pyruvate, or cAMP had no apparent effect on the rate of acetyl-CoA hydrolysis (Prass et al., 1980). The hydrolytic activity was relatively specific for short-chain acyl-CoA: longer chain acyl-CoA compounds were hydrolyzed more slowly, and enzyme affinity increases with the chain length, suggesting that longer chain CoAs may be competitive inhibitors (Prass et al., 1977, 1980). CoASH is also possibly an inhibitor (Prass et al., 1977, 1980). Palmityl-CoA probably causes nonspecific inhibition, while malonyl-CoA is neither a substrate nor an inhibitor (Prass et al., 1980).

To study the effects of nucleotides on ATP-stimulated, ADP-inhibited acetyl-CoA hydrolase, we developed a method for purification of the enzyme in the absence of nucleotides and eventually achieved a homogeneous enzyme preparation. This paper reports the effects of nucleotides and temperature on the molecular structure of the purified enzyme and that nucleotides regulate not only the catalytic properties of this enzyme but also the association–dissociation state of subunits of this oligomeric enzyme. A preliminary report of part of this work has been presented (Isohashi et al., 1981).

## Materials and Methods

*Materials.* CoASH, ATP, ADP, AMP, ITP, UTP, CTP, GTP, TTP, PMSF, DTT, BSA, $\gamma$-globulin, and pyruvate kinase were obtained from Sigma Chemical Co. DEAE-Sephadex A-50, CM-Sephadex C-50, and blue dextran 2000 were purchased from Pharmacia Fine Chemicals. Bio-Gel HTP and Bio-Gel A-1.5m were obtained from Bio-Rad Laboratories. Molecular weight marker (horse heart myoglobin) for sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis was obtained from BDH Co., Ltd. Nonenzymatic protein molecular weight markers for molecular weight determination by gel filtration were purchased from Schwarz/Mann. Acetyl-CoA was prepared from CoASH and acetic anhydride as reported previously (Simon & Shemin, 1953). Unless otherwise specified, all other chemicals (reagent grade) were purchased from Wako Pure Chemical Industries, Ltd.

*Animals and Treatment.* Male albino Donryu rats, weighing 55–65 g, were kept under standard laboratory conditions (Isohashi et al., 1976) and were used for experiments when they weighed 300–400 g.

*Assay of Acetyl-CoA Hydrolase.* Enzyme activity was routinely assayed at 25 °C by determining the rate of formation of CoASH from acetyl-CoA using 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as reported previously (Prass et al., 1980). When specified, enzyme was assayed at 30 °C.

*Buffers.* Buffer A consisted of 0.25 M sucrose, 0.02% $NaN_3$, 1 mM PMSF, 2 mM DTT, and 10 mM $KH_2PO_4$, adjusted to pH 7.2 with KOH. Buffer B (pH 7.2) consisted of buffer A with 50 mM $KH_2PO_4$, rather than 10 mM $KH_2PO_4$. Buffer C (pH 7.2) consisted of buffer A with 200 mM $KH_2PO_4$. Buffer D (pH 7.2) consisted of buffer A with 100 mM $KH_2PO_4$ and 0.5 mM EDTA. Buffer E (pH 7.2) consisted of buffer A with 25 mM $KH_2PO_4$ and 0.5 mM EDTA without sucrose. Buffer F (pH 7.2) consisted of buffer A without sucrose. Buffer G (pH 7.2) consisted of buffer A with 100 mM $KH_2PO_4$, 2.3 M sucrose, and 1 mM EDTA. Buffer H (pH 7.2) consisted of buffer D without sucrose.

*Purification of Acetyl-CoA Hydrolase.* Unless otherwise specified, all procedures were carried out at room temperature (25 °C). Acetyl-CoA hydrolase was purified by the method of Prass et al. (1980) with the following modifications.

*Step I: Preparation of Supernatant Fraction.* Livers (350 g) from rats that had been starved for 48 h were homogenized with 2 volumes of buffer A, and the homogenate was centrifuged at 21000*g* for 10 min. The resulting supernatant was centrifuged at 105000*g* for 30 min to obtain the supernatant fraction (600 mL).

*Step II: Hydroxylapatite Treatment.* Hydroxylapatite (Bio-Gel HTP) was equilibrated with buffer A. The sample was mixed with 600 mL of hydroxylapatite slurry with gentle stirring for 30 min. Then the slurry was stood overnight at 25 °C and centrifuged at 850*g* for 5 min. The pellet was washed twice with 600 mL of buffer B and extracted 3 times with 600 mL of buffer C. The three extracts were combined.

*Step III: Ammonium Sulfate Fractionation.* This procedure was carried out according to the method reported previously (Prass et al., 1980) except ammonium sulfate concentration. The fraction from 20 to 33% saturation of ammonium sulfate was collected.

*Step IV: Heat Treatment.* The collected sample was adjusted to 40% saturation of ammonium sulfate. The resulting pellet was dissolved in buffer D and adjusted to a protein concentration to about 12 mg/mL. The sample was incubated at 50 °C for 25 min, then rapidly cooled to 25 °C, and centrifuged at 21000*g* for 10 min to obtain a clear supernatant.

*Step V: DEAE-Sephadex A-50 Column Chromatography.* After heat treatment, the sample was adjusted to 40% saturation of ammonium sulfate. The resulting pellet was suspended in buffer E, dialyzed against buffer E for 4 h, and then applied to a DEAE-Sephadex A-50 column (3 × 14.3 cm) equilibrated with buffer E. The column was washed with 150 mL of buffer E containing 0.25 M NaCl, and then the enzyme was eluted with buffer E containing 0.4 M NaCl.

*Step VI: CM-Sephadex C-50 Column Chromatography.* The sample from step V was adjusted to 40% saturation of ammonium sulfate, and the resulting pellet was resuspended in buffer F and dialyzed against the same buffer for 4 h. The dialyzed sample was applied to a CM-Sephadex C-50 column (3 × 7 cm) equilibrated with buffer F, washed with 50 mL of buffer F, and eluted with 500 mL of a linear gradient of 0–1 M KCl in the same buffer. The peak fractions, which showed almost identical specific activity, were combined and concentrated to 0.1 mL in a single hollow fiber concentrator (MDA Scientific, Inc., Park Ridge, IL). The concentrated sample was suspended in 0.2 mL of buffer G and stored at −80 °C, or at 25 °C when the sample was to be used within a few days.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis.* $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out by the method of Weber & Osborn (1969), except that the

Table I: Purification of Acetyl-CoA Hydrolase from Rat Liver[a]

| fraction | total protein (mg) | total act. (μmol/min) | sp act. (units/mg) | purification (x-fold) | yield (%) |
|---|---|---|---|---|---|
| homogenate | 59700 | 4800 | 0.08 | 1 | 100 |
| supernatant (105000g, 30 min) | 20400 | 3100 | 0.15 | 1.9 | 65 |
| hydroxylapatite eluate | 8800 | 2800 | 0.32 | 4.0 | 58 |
| $(NH_4)_2SO_4$ fractionation (20–33%) | 1100 | 2400 | 2.2 | 28 | 50 |
| heat treatment | 380 | 1900 | 5.0 | 63 | 40 |
| DEAE-Sephadex eluate | 71 | 1100 | 16 | 200 | 23 |
| CM-Sephadex eluate, concentrated | 1.7 (0.8)[b] | 630 (790)[c] | 370 (990)[d] | 4600 | 13 |

[a] ATP-stimulated, ADP-inhibited acetyl-CoA hydrolase was purified from rat liver at 25 °C. The purification procedures are described under Materials and Methods. Unless otherwise specified, the enzyme activity was determined at 25 °C by spectrophotomeric assay by subtracting the activity measured with 2 mM ADP from that measured with 2 mM ATP. The activity of the final concentrated product was assayed at 25 and 30 °C. Protein was determined by a modification (Hartree, 1972) of the method of Lowry et al. (1951). Protein in the final concentrated sample was determined with this method and with Coomassie blue (Bradford, 1976). [b] Protein determined with Coomassie blue. [c] Enzyme activity assayed at 30 °C. [d] Specific activity was calculated by dividing the activity at 30 °C by the protein content determined with Coomassie blue.

gel contained 5% acrylamide. The subunit molecular weight of acetyl-CoA hydrolase was estimated by $NaDodSO_4$–polyacrylamide gel electrophoresis by comparison with molecular weight standards [horse heart myoglobin: monomer ($M_r$ 14300), dimer ($M_r$ 28600), trimer ($M_r$ 42900), tetramer ($M_r$ 57200), pentamer ($M_r$ 71500), and hexamer ($M_r$ 85800)].

*Gel Filtration.* A Bio-Gel A-1.5m column was equilibrated with buffer D with or without 0.5 mM ATP, 0.5 mM ADP, or 0.5 mM AMP. Concentrated samples were adjusted to a protein concentration of 0.5 mg/mL by addition of buffer D with or without 2 mM ATP, ADP, or AMP and incubated for 1 h at 25 °C. The samples (about 50 μg/100 μL) were then applied to a column and eluted with the equilibration buffer at a rate of 4 mL/h at 25 °C. Columns were calibrated with pyruvate kinase ($M_r$ 237000), γ-globulin ($M_r$ 160000), BSA ($M_r$ 67000), ovalbumin ($M_r$ 45000), and myoglobin ($M_r$ 17200).

*Sucrose Density Gradient Centrifugation.* Sucrose solutions were prepared in buffer H. After storage, the purified enzyme was diluted with buffer H to 0.6 mg of protein/mL, and an aliquot of 0.3 mL was placed on the top of a linear 10–20% sucrose gradient (5 mL) at 4 °C or a 5–35% sucrose gradient (5 mL) at 25 °C and centrifuged at 120000g for 20 h in a Beckman L-2 centrifuge at 4 or 25 °C. The molecular weight markers used were BSA [$M_r$ 67000 (4.6 S)] and γ-globulin [$M_r$ 160000 (7.0 S)].

*Cold Lability.* The purified enzyme (4 mg/mL) was diluted 300 times with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mg/mL BSA with or without 2 mM of the various nucleotides indicated in Table II. Aliquots were incubated at different temperatures for various times, and then remaining activities were measured.

*Protein Determination.* Protein was determined by a modification (Hartree, 1972) of the method of Lowry et al. (1951) with BSA as a standard or, when specified, by the method of Bradford (1976).

## Results

*Purification of ATP-Stimulated, ADP-Inhibited Acetyl-CoA Hydrolase from the Supernatant Fraction of Rat Liver.* Extramitochondrial acetyl-CoA hydrolase is known to be extremely labile at low temperature and fairly stable at 25 °C (Prass et al., 1980). Thus, all purification procedures were carried out at 25 °C. To examine the effects of nucleotides on the enzyme, we purified it without using ATP as a stabilizer. A typical purification procedure is summarized in Table I. The yield after concentration of the pooled fraction from CM-Sephadex was about twice that reported previously (Prass et al., 1980), while the specific activity was comparable to that reported (Prass et al., 1977, 1980).

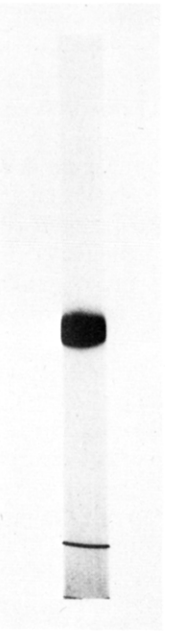

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of acetyl-CoA hydrolase. $NaDodSO_4$–polyacrylamide gel electrophoresis was carried out as described by Weber & Osborn (1969). About 30 μg of purified acetyl-CoA hydrolase was loaded on a 5% gel.

*$NaDodSO_4$–Polyacrylamide Gel Electrophoresis and Subunit Molecular Weight.* As shown in Figure 1, the purified enzyme gave a single protein band on 5% $NaDodSO_4$–polyacrylamide gel electrophoresis. The enzyme was judged to be more than 90% pure by scanning this $NaDodSO_4$–polyacrylamide gel. The subunit molecular weight was estimated as 63000 by 5% $NaDodSO_4$–polyacrylamide gel electrophoresis as described under Materials and Methods.

*Gel Filtration and Molecular Weight.* The molecular weight of the purified enzyme was estimated by gel filtration (Bio-Gel A-1.5m) as 135000 (Figure 2), suggesting that it consists of two subunits of identical molecular weight ($M_r$ 63000) (Figure 1). Unexpectedly, this value is about half that reported previously ($M_r$ 240000, at least four subunits of identical molecular weight) (Prass et al., 1977, 1980). To clarify this apparent discrepancy between the two purified samples, we prepared the purified enzyme (tetramer) by the procedures used previously (Prass et al., 1980) and then dialyzed it against buffer D without ATP and subjected the dialyzed sample to gel filtration. Results showed that dialysis of the enzyme of larger molecular weight ($M_r$ 240000, four subunits) against buffer D resulted in formation of enzyme

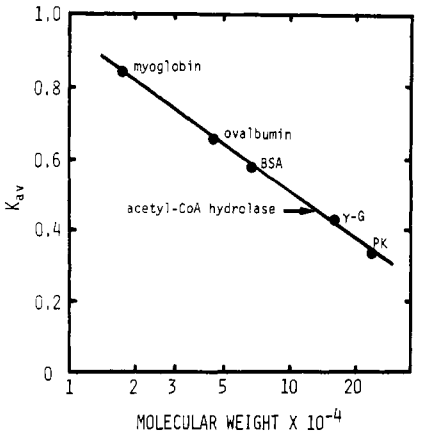


FIGURE 2: Determination of the molecular weight of purified acetyl-CoA hydrolase by gel filtration. The molecular weight of the purified enzyme was determined by gel filtration on Bio-Gel A-1.5m. A column (1 × 38 cm) of Bio-Gel A-1.5m was equilibrated with buffer D. The purified sample diluted to a protein concentration of about 50 μg/100 μL with buffer D was applied to the column and eluted with the same buffer. The column was calibrated with pyruvate kinase (PK; $M_r$ 237 000), γ-globulin (γ-G; $M_r$ 160 000), bovine serum albumin (BSA; $M_r$ 67 000), ovalbumin ($M_r$ 45 000), and myoglobin ($M_r$ 17 200). The molecular weight was estimated by interpolation from a standard plot of the log molecular weight against $K_{av}$. $K_{av} = (V_e - V_0)/(V_t - V_0)$ where $V_e$ is elution volume, $V_0$, void volume, and $V_t$, column volume.

of smaller molecular weight ($M_r$ 135 000, two subunits), suggesting that this molecular weight shift resulted from removal of ATP from the purified sample by dialysis.

*Reconstitution or Aggregation of the Dimeric Enzyme to the Tetrameric Enzyme.* This idea was tested by reconstitution studies using the purified dimeric enzyme ($M_r$ 135 000). The purified enzyme was treated with 2 mM ATP at 25 °C for 1 h and then applied on a Bio-Gel A-1.5m column equilibrated with buffer D containing 0.5 mM ATP and eluted with the same buffer (Figure 3B). The molecular weight of the major protein peak of ATP-treated enzyme moved from about 135 000 (Figure 3A) to about 242 000 (Figure 3B), suggesting that ATP associates or aggregates the dimeric form to the tetrameric form reversibly.

Next, similar experiments were done with ADP (Figure 3C) and AMP (Figure 3D). In the presence of ADP and AMP, the molecular weights of the major protein peaks of the enzyme were 230 000 and 145 000, respectively, suggesting that ADP can also associate or aggregate the dimeric enzyme to the tetramer but that AMP cannot. The shoulder or the minor protein peak of the enzyme, nearly corresponding to the position of bovine serum albumin seen in Figure 3A,D, appeared to be the monomeric form of the enzyme dissociated from the dimeric enzyme. Addition of ATP (Figure 3B) or ADP (Figure 3C) decreased the proportions of these protein peaks, suggesting a shift from the monomeric enzyme to the larger molecular weight enzyme or protection against dissociation of dimeric enzyme into a smaller molecule.

*Effect of ATP on Acetyl-CoA Kinetics of Various Forms of the Enzymes.* There is a report that acetyl-CoA hydrolase (tetramer) is highly regulated by nucleotides and that the presence of 2 mM ATP in the assay medium lowers the apparent $K_{0.5}$ for acetyl-CoA from 1.3 mM (no nucleotide) to 60 μM, while 2 mM ADP completely inhibits the enzymatic activity (Prass et al., 1980). In the absence of ATP, acetyl-CoA kinetics exhibits negative cooperativity ($n_H$ = 0.74) but is linear ($n_H$ = 1.0) with 2 mM ATP (Prass et al., 1980). It is interesting that the dimeric enzyme without ATP also hydrolyzed acetyl-CoA at a rate over the nonenzymatic level.

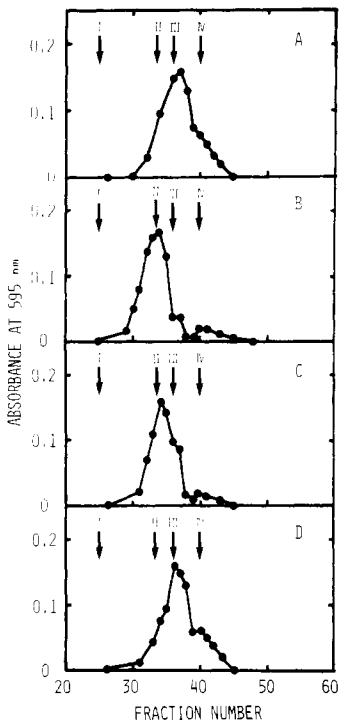


FIGURE 3: Effects of nucleotides on the molecular structure of acetyl-CoA hydrolase. The molecular weight of acetyl-CoA hydrolase was estimated by gel filtration on Bio-Gel A-1.5m. See Materials and Methods and the legend for Figure 2 for details of the method. Bio-Gel A-1.5m columns were equilibrated with buffer D without nucleotide (A) or with 0.5 mM ATP (B), 0.5 mM ADP (C), or 0.5 mM AMP (D). A concentrated sample (purified dimeric enzyme) was adjusted to a protein concentration of 0.5 mg/mL with buffer D with or without 2 mM ATP, ADP, or AMP and incubated for 1 h at 25 °C. Samples (about 50 μg/100 μL) were then applied to columns and eluted with equilibration buffer at a rate of 4 mL/h at 25 °C. Fractions of 0.6 mL were collected. Columns were calibrated with blue dextran 2000 (I, void volume), pyruvate kinase (II; $M_r$ 237 000), γ-globulin (III; $M_r$ 160 000), and bovine serum albumin (IV, $M_r$ 67,000). Protein was stained with Coomassie blue and measured spectrophotometrically at 595 nm (Bradford, 1976).

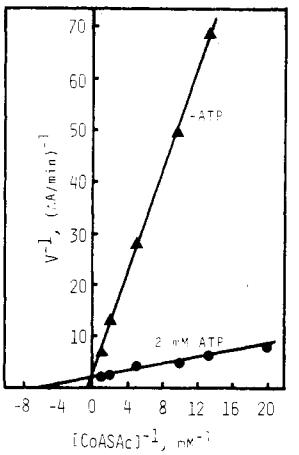


FIGURE 4: Lineweaver–Burk plot of the activity of the purified acetyl-CoA hydrolase (dimer) with or without ATP. Enzyme activities were measured at 30 °C in assay mixtures with various concentrations of acetyl-CoA with (●) or without (▲) 2 mM ATP.

Lineweaver–Burk plots of the activities of dimeric enzyme with various concentrations of the substrate in the absence of ATP were linear (Figure 4), indicating no cooperativity ($n_H$ = 1.0 determined by a Hill plot). Addition of 2 mM ATP to the assay medium, however, increased the enzymatic activity 5.3-fold at 0.5 mM acetyl-CoA and lowered the apparent $K_m$ for acetyl-CoA from 2.2 mM (no ATP) to 170 μM without

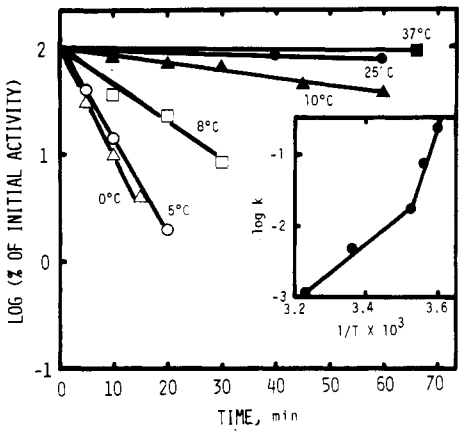


FIGURE 5: Effect of temperature on the rate of inactivation of purified acetyl-CoA hydrolase (dimer). The purified dimeric enzyme (4 mg/mL) was diluted 300 times with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mg/mL bovine serum albumin and incubated at different temperatures: (■) 37, (●) 25, (▲) 10, (□) 8, (○) 5, and (△) 0 °C. Samples of incubation mixtures were taken at intervals for measurement of the remaining activity at 25 °C in the presence of 2 mM ATP. Activity immediately after dilution of the purified enzyme at 25 °C was taken as 100%. The insert shows a log plot of the inactivation rate constant ($k$) at various temperatures vs. the reciprocal of the absolute temperature of incubation.

an appreciable change in the $V_{max}$ (Figure 4). Addition of 2 mM ADP reduced the activity of the dimeric enzyme to the nonenzymatic level. Furthermore, in the presence of 2 mM ATP, the hydrolytic rates of the two types of enzyme (dimeric and tetrameric forms) were similar on the basis of the purified enzyme protein contents, although the apparent $K_m$ value (170 $\mu$M) of the dimeric enzyme was higher than that of the tetrameric enzyme (60 $\mu$M). When the dimeric enzyme was converted to the tetrameric enzyme (Figure 3B), the apparent $K_m$ for acetyl-CoA was reduced to 60 $\mu$M (data not shown). Under the assay conditions with 2 mM ATP and a low concentration of protein, there was no evidence of association or aggregation of the dimeric enzyme to the tetrameric one. In fact, the initial activity of the dimeric enzyme in medium with 2 mM ATP showed no lag and no shift in the rate of the activity, and hydrolysis of acetyl-CoA gave a linear Michaelis–Menten type plot with a $K_m$ value of 170 $\mu$M for acetyl-CoA instead of that of 60 $\mu$M with the tetrameric enzyme.

*Effect of Temperature on the Rate of Inactivation of Purified Acetyl-CoA Hydrolase.* Acetyl-CoA hydrolase is known to be rapidly inactivated at 4 °C ($t_{1/2}$ = 10 min) in a crude homogenate of rat liver (Prass et al., 1980). The enzyme in excised whole liver also shows extreme cold lability (Isohashi et al., 1981). However, it is unknown whether this cold lability is a property of the enzyme itself or is caused by some factors in the crude preparation. To test this, we incubated highly purified enzyme (dimeric form) without ATP at various temperatures. Figure 5 shows that the purified enzyme was fairly stable at above 25 °C during the experimental period, but that at lower temperatures, especially below 10 °C, it was rapidly inactivated with $t_{1/2}$ values of approximately 40 min at 10 °C, 9 min at 8 °C, 3.4 min at 5 °C, and 3.0 min at 0 °C (Figure 5). Thus cold lability seems to be an inherent property of the enzyme. The insert in Figure 5 shows log plots of the inactivation rate constant ($k$) at various temperatures vs. the reciprocal of the absolute temperature of incubation. This plot suggests two successive first-order kinetics: early slow and later faster inactivation. The transition point is at about 10 °C, below which more marked cold inactivation was demonstrable under the experimental conditions.

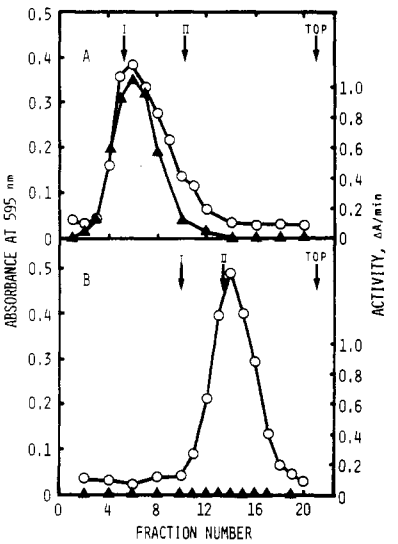


FIGURE 6: Sucrose density gradient centrifugation. The stored purified enzyme was adjusted to a protein concentration of about 0.6 mg/mL with buffer H. A sample of 0.3 mL was placed on top of a linear gradient of 5–35% sucrose (5 mL) at 25 °C (A) or of 10–20% sucrose (5 mL) at 4 °C (B) and centrifuged at 120000$g$ for 20 h at 25 (A) or 4 °C (B), respectively. Protein (○) was stained with the Coomassie blue and measured at 595 nm (Bradford, 1976). The enzyme activity (▲) was measured at 25 °C in the presence of 2 mM ATP. The markers used were (I) γ-globulin ($M_r$ 160000) (7.0 S) and (II) BSA ($M_r$ 67000) (4.6 S).

Table II: Effects of Various Nucleotides on Cold Inactivation of Acetyl-CoA Hydrolase[a]

| | residual activity after incubation | | | |
|---|---|---|---|---|
| | 8 °C | | 0 °C | |
| addition | 10 min (%) | 30 min (%) | 10 min (%) | 30 min (%) |
| none | 35 | 8 | 10 | 2 |
| ATP | 91 | 69 | 60 | 26 |
| ADP | 73 | 51 | 21 | 6 |
| AMP | 53 | 21 | 11 | 4 |
| ITP | 79 | 51 | 36 | 11 |
| UTP | 79 | 52 | 38 | 12 |
| CTP | 61 | 32 | 20 | 6 |
| GTP | 58 | 22 | 12 | 5 |
| TTP | 58 | 25 | 13 | 6 |

[a] The purified dimeric enzyme (4 mg/mL) was diluted 300 times with 100 mM potassium phosphate buffer (pH 7.8) containing 1 mg/mL bovine serum albumin with or without 2 mM nucleotide. The diluted samples were incubated at 8 or 0 °C for 10 or 30 min. Then samples were taken for measurement of activity at 25 °C in the presence of 2 mM ATP. Samples incubated at 25 °C with or without nucleotides were stable during the experimental period. Activity determined immediately after dilution at 25 °C was taken as 100%.

*Effect of Cold on Sedimentation of Highly Purified Dimeric Enzyme.* On linear sucrose density gradient centrifugation at 25 °C, the purified enzyme (dimeric form) gave a protein peak coinciding with activity at about 6.6 S (Figure 6A). However, at 4 °C, the enzyme protein peak was at 4.1 S where little enzyme activity was detected (Figure 6B). These results suggest that the dimeric form of enzyme at 25 °C dissociated into a smaller molecule at 4 °C which has a molecular weight similar to that of the subunit of the enzyme [$M_r$ 63000 determined by $NaDodSO_4$–polyacrylamide gel electrophoresis (Figure 1)] and that the protomeric form has no catalytic activity.

*Effects of Various Nucleotides on Inactivation by Low Temperature.* As described in Table II, the purified enzyme

(dimeric form) was protected to some extent against inactivation at low temperature by ATP. As described earlier, at 0 °C the dimeric enzyme was inactivated with a $t_{1/2}$ value of about 3.0 min without ATP. However, in separate experiments, addition of 2 mM ATP decreased in the rate of inactivation at 0 °C with a $t_{1/2}$ value of about 14.3 min. Table II also shows that ADP, ITP, and UTP prevented inactivation by cold though less than ATP. AMP, CTP, GTP, and TTP had only slight protective effects.

*Effects of Cold on the Sedimentation Properties of ATP-Treated Enzyme (Reconstituted Tetramer) in the Presence of ATP.* As described above, ATP could associate the dimeric enzyme to tetramer at 25 °C (Figure 3) and also partially prevent inactivation of the dimeric enzyme by cold (Table II). Thus, it was of particular interest to determine the dissociation–association state of ATP-treated enzyme [tetramer at 25 °C (Figure 3)] in the presence of 2 mM ATP at low temperature. Linear sucrose density gradient centrifugation at 4 °C showed that in the presence of ATP, the tetrameric enzyme dissociated to monomer at low temperature, losing its catalytic activity (data not shown).

## Discussion

Extramitochondrial acetyl-CoA hydrolase in rat liver is very labile in a crude homogenate with cold isotonic buffer (Prass et al., 1980) and in whole liver at low temperature (Isohashi et al., 1981), which is routinely used for tissue manipulation in biochemical studies. Thus this enzyme has been demonstrated only recently (Prass et al., 1977, 1980). The purification procedures reported previously (Prass et al., 1980) and in this paper were conducted at room temperature (25 °C), the former with ATP as an enzyme stabilizer and the latter without ATP. The final products obtained by the two methods had similar specific activity and were found to be more than 90% pure and to have a similar protomeric molecular weight as judged by $NaDodSO_4$ gel electrophoresis. The intact purified enzyme, however, is known to have a molecular weight of 240 000 and to be composed of four subunits of identical molecular weight (Prass et al., 1977, 1980), while the enzyme reported in this paper had a molecular weight of 135 000 and two subunits. This discrepancy led us to discover that ATP has a key role in the association–dissociation state of the enzyme. In fact, the tetrameric enzyme was obtained in the presence of ATP (Prass et al., 1980), and addition of ATP to the concentrated dimeric enzyme at 25 °C caused its association or aggregation to the tetrameric form (Figure 3). Furthermore, when ATP was removed from the ATP-treated tetrameric enzyme by dialysis, the tetramer was mostly dissociated into the dimeric form. It is interesting that addition of ADP, which is an inhibitor of this enzyme, to the concentrated dimeric enzyme at 25 °C also resulted in formation of tetramer though without catalytic active. Addition of AMP did not affect the aggregation state of the enzyme or its activity, but the dimeric enzyme could be reversibly associated with AMP–Sepharose.[3]

In assay medium containing 2 mM ATP, there was about 5-fold increase in the enzyme activity on 0.5 mM acetyl-CoA of the tetrameric and dimeric enzyme with similar specific activity. However, the $K_m$ values for acetyl-CoA of the dimeric and tetrameric enzyme were different, being 170 μM and 60 μM, respectively. Thus, ATP appears to have effects on the catalytic properties of acetyl-CoA hydrolase and on the association–dissociation state of the enzyme. The change from the dimeric to the tetrameric state may increase the affinity for the substrate and be related to regulation of the cytosolic CoASH or acetyl-CoA level, although the physiological role of this enzyme is unknown. In assay medium with 2 mM ADP, neither the tetrameric nor dimeric enzyme showed enzymatic activity. Thus, ADP may also have effects on both the catalytic properties and aggregation state of the enzyme and may be important in regulation of the hydrolytic rate.

The enzyme in crude preparations is extremely cold labile (Prass et al., 1980), and at low protein concentration the dimeric and tetrameric forms of purified enzyme are also dissociated at low temperature to monomer with no activity and no reactivation at 25 °C. However, recently partial reactivation by rewarming was observed with more concentrated enzyme, as will be reported elsewhere. Addition of ATP and other nucleotides partially prevented cold inactivation of the purified dimeric enzyme. The failure to detect these effects of nucleotides in crude systems in earlier studies (Prass et al., 1980) may be explained by the presence of sufficient contaminating nucleotide to prevent cold inactivation, so that addition of ATP or ADP had no further protective effect. It is noteworthy that in the presence of 2 mM ATP, the inactivation rates of the dimeric and tetrameric enzymes at 0 °C had similar first-order kinetics and half-times of 5 times that of the dimeric enzyme without nucleotides. If the tetramer dissociates to the monomer via the dimer, this result suggests that the rate-limiting step in cold inactivation or dissociation is mainly, if not entirely, conversion of the dimeric form to the monomer and that ATP affects the dimer–monomer equilibrium at 0 °C, although ATP also affected the dimer–tetramer interconversion at 25 °C.

We conclude from this work that nucleotides regulate not only the catalytic properties of extramitochondrial acetyl-CoA hydrolase but also the association–dissociation state of subunits of this enzyme. This oligomeric enzyme has high activity levels in rat liver (Prass et al., 1980), marked substrate specificity (Prass et al., 1980), and the complicated manner of regulation, suggesting that the enzyme has a significant physiological role.

## Acknowledgments

We dedicate this paper to Dr. Merton F. Utter, who died on Nov 28, 1980. We thank Dr. T. Higashi, Dr. K. Tsukanaka, Dr. M. Terada, Dr. T. Matsunaga, Dr. M. Horiuchi, Dr. K. Okamoto, and Dr. Y. Mitsui, Osaka University Medical School, for helpful discussions and encouragement. We also thank H. Ohno, K. C. Park, and C. Ueta, undergraduate students of Osaka University Medical School, for technical assistance and M. Fukuoka for secretarial assistance.

**Registry No.** ATP, 56-65-5; ADP, 58-64-0; AMP, 61-19-8; ITP, 132-06-9; UTP, 63-39-8; CTP, 65-47-4; GTP, 86-01-1; TTP, 365-08-2; acetyl-CoA, 72-89-9; acetyl-CoA hydrolase, 9027-54-7.

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# Membrane-Bound Kidney Neutral Metalloendopeptidase: Interaction with Synthetic Substrates, Natural Peptides, and Inhibitors†

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ABSTRACT: A neutral metalloendopeptidase with a thermolysin-like specificity was purified to apparent homogeneity, from the particulate fraction of rabbit kidney homogenates. After preparration of a deoxycholate extract, the enzyme was released from membranes by papain treatment and separated from other membrane-bound enzymes including dipeptidyl aminopeptidase IV, aminopeptidase M, and γ-glutamyl transpeptidase by chromatography on Sephadex G-200, phenyl-Sepharose, and carboxymethylcellulose columns. The isolated enzyme has a molecular weight of about 95 000 and is inhibited by thiols, metal chelators, phosphoramidon, and thiorphan. It is apparently identical with the kidney neutral metalloendopeptidase (EC 3.4.24.11) [Kerr, M. A., & Kenny, A. J. (1974) *Biochem. J. 137*, 447–488] and similar to the bovine pituitary metalloendopeptidase [Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942–4950] and to an enzyme designated as "enkephalinase". Studies with a series of synthetic substrates showed that the enzyme preferentially cleaves bonds in which the amino group is provided by a hydrophobic amino acid residue. Several biologically active peptides such as methionine and leucine enkephalin, dynorphin, bradykinin, and angiotensin I are degraded by cleavage of the same type of bond. The endopeptidase acts as a dipeptidyl carboxypeptidase on peptides having a hydrophobic residue in the penultimate position. *N*-[1(*R*,*S*)-Carboxy-2-phenylethyl] derivatives of phenylalanyl- and alanyl-*p*-aminobenzoate were synthesized and tested as potential inhibitors. The two diastereomers of *N*-[1(*R*,*S*)-carboxy-2-phenylethyl]phenylalanyl-*p*-aminobenzoate were separated by high-pressure liquid chromatography; the more potent isomer had a $K_I$ of $2.9 \times 10^{-8}$ M. The inhibitory potency of the alanyl derivatives was lower by almost 2 orders of magnitude. The data indicate that, as with thermolysin, a hydrophobic residue in the $P_1'$ position and the carboxylate group complexing with the active site zinc atom account for the inhibitory action of these derivatives.

We have previously reported the purification from bovine pituitaries of a membrane-bound metalloendopeptidase with a specificity directed toward peptide bonds in which the amino group is contributed by hydrophobic amino acid residues (Orlowski & Wilk, 1981). Subsequent studies in our laboratory have shown that this thermolysin-like endopeptidase is apparently identical with "enkephalinase", an enzyme associated with brain membrane fractions, which cleaves the Gly–Phe bond in Met-enkephalin (Tyr-Gly-Gly-Phe-Met) and in Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) (Almenoff et al., 1981). There is a growing interest in enzymes that degrade enkephalins and other biologically active peptides, since their action may control the function of these potent substances. Furthermore, inhibition of enkephalin degradation could induce morphinomimetic effects of potential pharmacological importance (Roques et al., 1980). Knowledge, therefore, of the specificity and mechanism of action of peptide metabolizing enzymes has both theoretical and practical significance.

We have previously reported that the pituitary enzyme bears resemblance to a neutral metalloendopeptidase (EC 3.4.24.11) isolated by Kerr & Kenny (1974a) from kidney brush border fractions. Recent work by these authors suggests that this enzyme, like the bovine pituitary metalloendopeptidase, is similar to brain enkephalinase (Fulcher et al., 1982).

The specific activity of the metalloendopeptidase in brain and pituitary is very low, making it difficult to isolate the enzyme from these tissues and impeding studies on its properties, specificity, and mechanism of action. This report describes a convenient procedure for isolation of the enzyme from rabbit kidney, a study of its specificity toward synthetic and natural peptides, and the effect of various inhibitors on enzyme activity. New carboxymethyl derivatives of Phe-pAB[1] were

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‡ J.A. is a trainee on Medical Scientist Training Grant GM-07280 from NIH.

0006-2960/83/0422-0590$01.50/0 