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Kinetic Studies of the Pyridoxal Kinase from Pig Liver: Slow-Binding Inhibition by Adenosine Tetraphosphopyridoxal

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ABSTRACT: Pyridoxal kinase from pig liver has been purified 10 000-fold to apparent homogeneity. The enzyme is a dimer of subunits of M_r 32 000. The enzyme is strongly inhibited by the product pyridoxal 5′-phosphate. Liver pyridoxamine phosphate oxidase, another enzyme involved in the biosynthesis of pyridoxal 5′-phosphate, is also strongly inhibited by this compound [Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089-2095]. Thus, the biosynthesis of pyridoxal 5′-phosphate in the liver might be regulated by the product inhibition of both pyridoxamine phosphate oxidase and pyridoxal kinase. Kinetic studies revealed that the catalytic reaction of liver pyridoxal kinase follows an ordered mechanism in which pyridoxal and ATP bind to the enzyme and ADP and pyridoxal 5′-phosphate are released from the enzyme, in this order. Adenosine tetraphosphopyridoxal was found to be a slow-binding inhibitor of pyridoxal kinase. Pre-steady-state kinetics of the inhibition revealed that the inhibitor and the enzyme form an initial weak complex prior to the formation of a tighter and slowly reversing complex. The overall inhibition constant was 2.4 μM. ATP markedly protects the enzyme against time-dependent inhibition by the inhibitor, whereas another substrate pyridoxal affords no protection. By contrast, adenosine triphosphopyridoxal is not a slow-binding inhibitor of this enzyme.

Pyridoxal kinase (EC 2.7.1.35) catalyzes the transfer of the γ-phosphate moiety of ATP to the 5-hydroxymethyl group of pyridoxal. This enzyme, in conjunction with pyridoxamine phosphate oxidase, is involved in the biosynthesis of PLP[1] (the coenzyme form for vitamin B_6 dependent enzymes) (Snell & Haskell, 1971). It has been reported that liver and brain pyridoxamine phosphate oxidases are strongly inhibited by the product PLP (Wada & Snell, 1961; Kwok & Churchich, 1980). This inhibition may be physiologically important for the regulation of PLP biosynthesis (Snell & Haskell, 1971; Lui et al., 1981). On the other hand, pyridoxal kinase has been purified from the brains of mammalian species (Neary & Diven, 1970; Kwok & Churchich, 1979a; Kerry et al., 1986)

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[1] Abbreviations: PLP, pyridoxal 5′-phosphate; AP_4-PL, adenosine tetraphosphopyridoxal; AP_3-PL, adenosine triphosphopyridoxal; AP_4-PN, adenosine tetraphosphopyridoxine.

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and characterized (Kwok & Churchich, 1979b; Churchich & Wu, 1982). None of these reports, however, describe the strong inhibition of this enzyme by PLP.

Kinetic analyses of brain pyridoxal kinase revealed that the mechanism is a random bi-bi mechanism (Churchich & Wu, 1981), suggesting that pyridoxal and ATP bind in close proximity with direct transfer of the γ-phosphate. We have recently synthesized adenosine polyphosphopyridoxals as affinity labeling reagents for several enzymes that contain a nucleotide-binding site (Tagaya & Fukui, 1986; Tagaya et al., 1987, 1988a,b; Noumi et al., 1987; Yagami et al., 1988; Yamamoto et al., 1988). Since adenosine tri- and tetraphosphopyridoxals contain pyridoxal and ATP moieties, these compounds may act as "bisubstrate analogues" and, therefore, strongly inhibit the enzyme [cf. Wolfenden (1972) and Lienhard (1972)].

In this paper, we describe the results of purification and kinetic analyses of pyridoxal kinases from pig, rabbit, and calf livers. We found that all of the liver enzymes are strongly inhibited by the product PLP, similar to the known sensitivity of liver and brain pyridoxamine phosphate oxidases to PLP (Wada & Snell, 1961; Kwok & Churchich, 1980). This finding suggests that the biosynthesis of PLP in the liver is regulated by feedback inhibition. The patterns of the product inhibition revealed that the reaction of pig liver pyridoxal kinase proceeds according to an ordered mechanism in which pyridoxal and ATP bind to the enzyme followed by ADP and PLP release from the enzyme, in this order. Furthermore, we found that AP_4-PL is a slow-binding inhibitor of this enzyme, whereas AP_3-PL is not.

Materials and Methods

Materials. ADP, ATP, malate dehydrogenase, and NADH were obtained from Oriental Yeast. Pyridoxal, PLP, L-aspartic acid, α-ketoglutaric acid, and Tris-HCl were obtained from Nakalai Tesque. DEAE-cellulose was obtained from Brown. AF-amino-Toyopearl 650M was purchased from Tosoh. Pyridoxyl-Toyopearl was prepared essentially by the method of Kerry et al. (1986). Sephadex G-100 was purchased from Pharmacia. AP_3-PL and AP_4-PL were synthesized by the method of Tagaya and Fukui (1986). Pig brain pyridoxal kinase was kindly donated by Dr. Jorge E. Churchich, Department of Biochemistry, University of Tennessee.

Preparation of Apoaspartate Aminotransferase. Aspartate aminotransferase was purified from *Escherichia coli* JM 103 harboring pKDHE essentially by the method of Kamitori et al. (1987). The enzyme thus prepared contained 28% holoenzyme and 72% apoenzyme. The prepared enzyme was treated with 10 mM $NaBH_4$ to inactivate the holoenzyme, then dialyzed against 50 mM Tris-HCl (pH 7.5) containing 50% glycerol, and stored at −20 °C.

Assay of Pyridoxal Kinase. The amount of PLP formed was determined (A) by using the apoaspartate aminotransferase (Yagi et al., 1985) or (B) by the absorbance at 410 nm derived from the Schiff base between PLP and Tris-HCl (Neary & Diven, 1970). One unit was defined as the amount of enzyme that produces 1 nmol of PLP/min under the conditions specified.

Method A. The assay mixture (0.99 mL, pH 6.1) contained 70 mM potassium phosphate, 0.5 M Tris-HCl, 1.0 mM pyridoxal, 1.0 mM ATP, and 0.5 mM $ZnCl_2$. The reaction was started by the addition of 10 μL of an enzyme solution, and the mixture was incubated at 37 °C. The reaction was stopped by heating the mixture in boiling water for 2 min, and then the mixture was centrifuged at 3000 rpm for 5 min. To 190 μL of 0.1 M Tris-HCl (pH 8.0) containing 0.07 μg/mL apoaspartate aminotransferase was added 10 μL of the supernatant. After incubation at 25 °C for 30 min, 100 μL of the solution was mixed with 1.7 mL of 0.1 M Tris-HCl (pH 8.0) containing 50 mM L-aspartic acid, 0.1 mM NADH, and 3.0 units of malate dehydrogenase. To this mixture was added 0.2 mL of 0.1 M Tris-HCl (pH 8.0) containing 50 mM α-ketoglutaric acid, and then the mixture was incubated at 25 °C. The decrease in absorbance at 340 nm was continuously monitored by use of a recording spectrophotometer.

Method B. The composition of the assay mixture was the same as that used in method A. The reaction was started by the addition of enzyme, and the absorbance at 410 nm was continuously monitored by use of a recording spectrophotometer. The absorption coefficient of the Schiff base was determined to be 5500 cm^{-1} M^{-1} at pH 6.1.

Purification of Pyridoxal Kinase. Unless otherwise stated, all the procedure was carried out at 4 °C. Pig liver (500 g) was thawed at 4 °C overnight, cut into small pieces, mixed with 3 volumes of 50 mM potassium phosphate (pH 6.9), and allowed to stand for 10 min. After homogenization, the crude extract was centrifuged at 10000*g* for 30 min, and the supernatant was passed through glass wool. To the filtrate was added pyridoxal to a final concentration of 1 mM, and then the mixture was incubated in a 55 °C water bath for 15 min, followed by cooling in an ice bath. After centrifugation at 10000*g* for 30 min, solid ammonium sulfate was added to the supernatant up to 40% saturation. After centrifugation at 10000*g*, more solid ammonium sulfate was added to the supernatant up to 60% saturation. The precipitate recovered by centrifugation was dissolved in 5 mM potassium phosphate (pH 7.4) containing 0.1% mercaptoethanol (buffer A). After the solution was dialyzed against 10 L of buffer A overnight with one change of the buffer, the protein solution was applied to a column (4.8 × 28 cm) of DEAE-cellulose, which had been equilibrated with buffer A. The column was washed with 1 L of buffer A and then eluted with a linear gradient composed of 1 L of buffer A (initial) and 1 L of buffer A containing 0.15 M KCl (limit), and fractions of 20 mL each were collected. Pyridoxal kinase activity was eluted as a broad peak. Fractions containing pyridoxal kinase were pooled and applied to a column (1.8 × 7 cm) of pyridoxyl-Toyopearl. The column was washed with 200 mL of buffer A containing 0.2 M KCl, followed by 100 mL of buffer A containing 0.5 M KCl. Pyridoxal kinase was eluted with 50 mM potassium phosphate (pH 5.0) containing 0.1% mercaptoethanol and 50 mM pyridoxal. The fractions containing enzymic activity were pooled, dialyzed, and concentrated to about 2 mL by ultrafiltration. The concentrated enzyme was applied to a column (1.6 × 86 cm) of Sephadex G-100 equilibrated with buffer A, developed with the same buffer, and fractions (3 mL each) containing enzyme activity were pooled, concentrated, and stored at 4 °C.

Polyacrylamide Gel Electrophoresis. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was carried out by the method of Laemmli (1970).

Protein Concentration. Protein concentration was determined by the method of Bradford (1976) by using bovine serum albumin as a standard.

Incorporation of PLP into Pyridoxal Kinase. PLP labeled at the 4-position with ^3H (46 200 cpm/nmol) was prepared as described previously (Maeda et al., 1988). Ten microliters of purified pyridoxal kinase (1 nmol) was mixed with 10 μL of 0.3 mM [^{3}H]PLP and 40 μL of 0.3 M potassium phosphate (pH 6.0). After incubation at 25 °C for 60 min, 10 μL of 0.1 M sodium borohydride and 40 μL of 10 M urea or water were successively added to the mixture. The protein was precipi-

Table I: Summary of Purification of Pig Liver Pyridoxal Kinase

step	protein (mg)	tota act. (units)	sp act. (units/mg)	yield (%)
homogenate	47 900	711	0.0148	100
heat treatment	29 300	624	0.0213	87.8
$(NH_4)_2SO_4$ fraction	11 900	728	0.0612	102
DEAE-cellulose	1 960	952	0.485	134
pyridoxyl-Toyopearl	1.49	145	97.3	20.4
Sephadex G-100	0.79	119	151	16.7

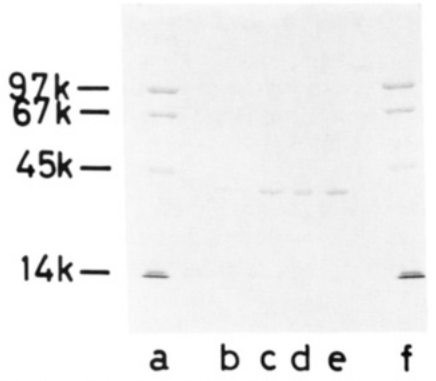

FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of pyridoxal kinase: molecular weight markers (lanes a and f), rabbit liver pyridoxal kinase (lane b), calf liver enzyme (lane c), a mixture of calf and pig liver enzymes (lane d), and pig liver enzyme (lane e).

tated in the presence of 0.18 mg/mL bovine serum albumin with 10% trichloroacetic acid. The precipitate was washed twice and then dissolved in 0.1 mL of 1 N NaOH. The solution was neutralized with 0.1 mL of 1 N HCl and mixed with 10 mL of scintillation cocktail. Radioactivity was measured with a Beckman Model 8900.

Slow Loss of Enzymic Activity. Pyridoxal kinase was incubated at 37 °C in 1 mL of potassium phosphate buffer (pH 6.1) containing 1.0 mM pyridoxal, 1.0 mM ATP, 0.5 mM $ZnCl_2$, 0.5 M Tris-HCl, and various concentrations of AP_4-PL, and absorbance at 410 nm was continuously monitored by use of a recording spectrophotometer.

Results

Purification and Molecular Weight. Pyridoxal kinase from pig liver was purified by ammonium sulfate fractionation and subsequent chromatographies on DEAE-cellulose, pyridoxyl-Toyopearl, and Sephadex G-100. A summary of the purification is shown in Table I. The purification fold was 10 000, and the final yield of the enzyme was 16.7% of the original activity. After the DEAE-cellulose chromatography step, an increase in the total enzymic activity by 1.3 times was observed. A similar increase in the activity was found in the purification of the pig brain enzyme (Kerry et al., 1986). The rabbit and calf enzymes were also purified by essentially the same method. All the enzymes thus purified were homogeneous by the criteria of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Figure 1). The subunit molecular weight of the pig liver enzyme was 32 000. The mobilities of the rabbit and calf enzymes were slightly larger than that of the pig enzyme (Figure 1). Gel filtration on Sephadex G-100 gave an estimated molecular weight for the native pig enzyme of 62 000, suggesting that the native enzyme is dimeric.

Inhibition by PLP. When enzymic activities of the purified pig liver pyridoxal kinase were measured by method B, except for the absence of Tris-HCl in the assay mixture, a nonlinear time course was observed (Figure 2a). Similar time courses were observed in the assay for the rabbit and calf enzymes (data not shown). Since pyridoxal kinase was stable at 37 °C,

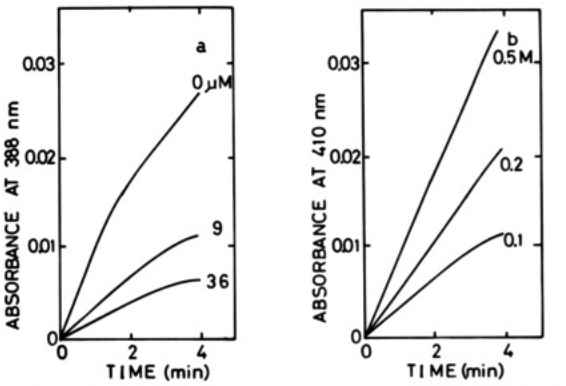

FIGURE 2: Time course of enzyme reaction. (a) Effect of PLP. Enzymic activity was measured in 70 mM potassium phosphate buffer (pH 6.1) containing 1.0 mM pyridoxal, 1.0 mM ATP, 0.5 mM Zn^{2+}, and various concentrations of PLP. Absorbance at 388 nm was continuously monitored. (b) Effect of Tris-HCl. Enzymic activity was measured in 70 mM potassium phosphate buffer (pH 6.1) containing 1.0 mM pyridoxal, 1.0 mM ATP, 0.5 mM Zn^{2+}, and various concentrations of Tris-HCl. Absorbance at 410 nm was continuously monitored.

the slow loss of enzymic activity may be due to the formation of an inhibitor during incubation. PLP, one of the products, at micromolar concentrations markedly inhibited the enzyme (Figure 2a), whereas another product, ADP, up to 50 μM did not affect the time course (data not shown). Pyridoxine phosphate at 50 μM did not inhibit the enzyme, suggesting that the formyl group of PLP is important for inhibition. Addition of Tris-HCl to the assay mixture suppressed the apparent inactivation, and the time course of enzymic activity was linear in the presence of 0.5 M Tris-HCl (Figure 2b). This is probably due to consumption of the formyl group by the formation of the Schiff base between PLP and Tris-HCl. The pig brain enzyme was not strongly inhibited by PLP (data not shown).

When pig liver pyridoxal kinase was preincubated with PLP, no change in the time course for enzymic activity was observed. Furthermore, no incorporation of PLP to the enzyme by treatment with sodium borohydride was observed when examined as described under Materials and Methods. These results suggest that PLP binds to the enzyme with a high affinity but does not form a Schiff base.

Kinetic Properties. The optimum pH for enzymic activity of pig liver pyridoxal kinase was 6.1. Among divalent cations, Zn^{2+} was the most effective activator. The relative enzymic activities measured in the presence of 0.5 mM amounts of various divalent cations compared with that measured in the presence of 0.5 mM Zn^{2+} were as follows: 95% (Co^{2+}), 67% (Mn^{2+}), 21% (Mg^{2+}), 15% (Cu^{2+}), 10% (Ca^{2+}), and 7% (Ni^{2+}). Among nucleoside triphosphates, ATP was the best substrate. The relative activities for various nucleotides at 1 mM compared with that for 1 mM ATP were as follows: 18% (GTP), 12% (UTP), and 8% (CTP).

Kinetic Mechanism. Since excess Zn^{2+} substantially inhibited the enzyme, enzymic activity was measured in 70 mM potassium phosphate (pH 6.1) containing 80 μM Zn^{2+} and various concentrations of pyridoxal and ATP. K_m values for pyridoxal and ATP were 125 μM and 59 μM, respectively. The double-reciprocal plots of the initial velocity against the concentration of pyridoxal or ATP gave a set of linear lines intercepting at a point on the x axis (data not shown), suggesting that this enzyme obeys a sequential (either random bi-bi or ordered) mechanism. To distinguish between the two mechanisms, we examined the effect of two products on the initial velocity. Enzymic activity was assayed in potassium

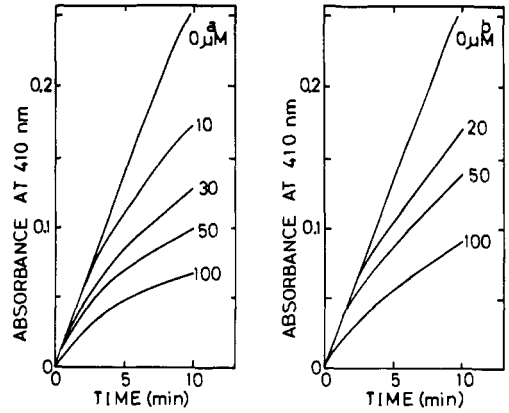

FIGURE 3: Progress curves of pyridoxal kinase assay. Pyridoxal kinase (0.05 nmol) was added to the assay mixtures containing AP_4-PL (a) or AP_4-PN (b). The concentrations of inhibitors are shown in the figure. Absorbance at 410 nm was continuously monitored.

phosphate buffer (pH 6.1) containing 160 μM Zn^{2+}, 80 μM ATP, and various concentrations of pyridoxal (20 μM pyridoxal when this substrate concentration was held constant) or various concentrations of ATP in the presence of either product. ADP showed noncompetitive inhibition with respect to both pyridoxal and ATP, whereas PLP (the Schiff derivative between PLP and Tris-HCl) inhibited the enzyme in competitive and noncompetitive manners, respectively, with pyridoxal and ATP (data not shown). These results suggest that pig liver pyridoxal kinase obeys an ordered mechanism in which pyridoxal and ATP bind to the enzyme and ADP and PLP are released from the enzyme, in this order (Cleland, 1970; Rudolph, 1979).

Inhibition of Pyridoxal Kinase by AP_4-PL. When pyridoxal kinase was assayed in the presence of AP_4-PL, a slow loss of enzymic activity was observed (Figure 3a). The inhibitory effect of AP_4-PL increased as its concentration increased. During these assay periods less than 4% of the original amounts of substrates were consumed, suggesting that the slow loss of enzyme activity is not due to the depletion of the substrates. These progress curves became linear after a long period (~40 min) at all the concentrations of the inhibitor, which suggest that the inhibition is reversible. Even if AP_4-PN lacking a reactive formyl group was substituted for AP_4-PL, similar patterns of inactivation were observed (Figure 3b). These results suggest that AP_4-PL neither covalently modifies nor reversibly inhibits the enzyme in a conventional manner. This type of inhibition is characteristic of slow-binding inhibition (Cha, 1975; Williams & Morrison, 1979).

Slow-Binding Mechanism for AP_4-PL. Analysis of the slow-binding patterns can be simplified if the following four conditions are satisfied (Williams & Morrison, 1979): (i) The amounts of substrates consumed during assay periods are less than 10% of the amounts originally added. (ii) The concentration of enzyme is much smaller than those of substrates. (iii) The concentration of the inhibitor does not significantly change during assay periods. In the present experiments, the concentration of pyridoxal kinase was 0.05 μM, significantly lower than those of substrates (1 mM pyridoxal and 1 mM ATP) and AP_4-PL (10–100 μM). Furthermore, the amounts of substrates consumed during assay periods were less than 4% of the amounts originally added, satisfying all the above three criteria. (iv) The last criterion is that the decrease in velocity during onset of inhibition follows the equation

$$v = v_s + [(v_0 - v_s) \exp(-k_{obsd}t)] \quad (1)$$

where v_s, v_0, and v are the steady-state, initial, and time t velocities, respectively, and k_{obsd} is the slope of a plot of ln ($v - v_s$) vs time. As shown in Figure 4a, the plot of ln ($v - v_s$) vs time gave a set of linear lines, satisfying the fourth criterion.

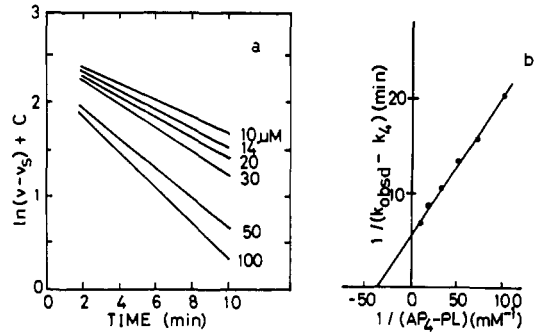

FIGURE 4: Determination of k_{obsd} for time-dependent inhibition of pyridoxal kinase by AP_4-PL. (a) Time-dependent inhibition by various concentrations of AP_4-PL. The concentrations of AP_4-PL are shown in the figure. (b) Plot of $1/(k_{obsd} - k_4)$ vs concentration of AP_4-PL.

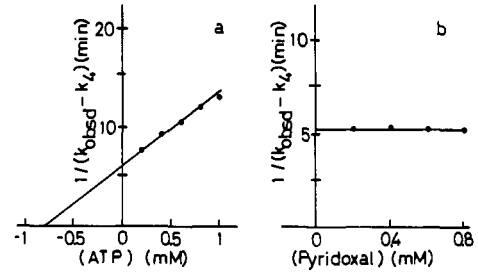

FIGURE 5: Effect of ATP (a) or pyridoxal (b) on time-dependent inhibition of pyridoxal kinase by AP_4-PL. The concentration of AP_4-PL was 30 μM.

If the slow binding proceeds according to the scheme

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E^*{\cdot}I$$

where E, I, and E* represent enzyme, inhibitor, and isomerized enzyme, respectively, the k_{obsd} value is

$$k_{obsd} = k_4 + k_3[I]/([I] + K_i') \quad (2)$$

where $K_i' = K_i(1 + [S]/K_m)$, $K_i = k_4/k_3$, and K_m is a Michaelis constant for ATP (Cha, 1975; Williams & Morrison, 1979). This equation predicts that the plot of $1/(k_{obsd} - k_4)$ vs $1/[I]$ gives a linear line intercepting at a positive value of the y axis and the value at interception on the x axis gives $-K_i'$. Since the rate of reactivation was too slow, it is difficult to measure a value for k_4. Therefore, we used the following relationship to obtain k_4 (Morrison, 1982):

$$k_4 = k_{obsd}v_s/v_0 \quad (3)$$

As shown in Figure 4b, the line intercepted at a positive value on the y axis. These results are consistent with the mechanism in which the enzyme and AP_4-PL initially form a rapidly reversible complex followed by isomerization of the complex.

We examined the effects of pyridoxal and ATP on the slow loss of enzymic activity. Interestingly, pyridoxal did not affect the slow binding of AP_4-PL, whereas ATP decreased the rate of apparent inactivation (Figure 5). This result suggests that the pyridoxal moiety of AP_4-PL does not occupy the pyridoxal-binding site of the enzyme in a pre steady state.

AP_3-PL was also tested to determine whether this reagent is a slow-binding inhibitor. However, no time-dependent loss of enzymic activity was observed when pyridoxal kinase was incubated with AP_3-PL up to 0.8 mM.

Discussion

We purified pyridoxal kinase from pig, rabbit, and calf livers to homogeneity and investigated the catalytic properties of the

pig enzyme. The liver enzyme is similar to the brain enzyme in molecular weight, optimum pH, requirement for divalent cations, and nucleoside triphosphate specificity (Kwok & Churchich, 1979a; Kerry et al., 1986). The K_m values of the liver enzyme for pyridoxal and ATP are slightly larger than those of the brain enzyme (Kerry et al., 1986) and similar to those of the sheep liver enzyme (Karawya & Fonda, 1978).

A remarkable difference between the two enzymes is the inhibition by the product PLP. Although the concentration of pyridoxal in the assay mixture is saturating, addition of 9 μM PLP decreased the enzyme activity by about 40% of that seen in its absence (Figure 2a). Strong inhibition by PLP was found not only in the pig liver enzyme but also in the rabbit and calf liver enzymes. The fact that PLP does not irreversibly inhibit the enzyme and that PLP is not incorporated into the liver enzyme after treatment with sodium borohydride suggests that Schiff base formation is not involved in binding. No strong inhibition by PLP was observed with brain pyridoxal kinase. Thus, this inhibition may be characteristic of the liver enzymes, with the exception of the sheep enzyme (Karawya & Fonda, 1978). It has been reported that pyridoxamine phosphate oxidase is strongly inhibited by the product PLP and its inhibition was suppressed by the addition of Tris-HCl to the assay mixture (Wada & Snell, 1961; Kwok & Churchich, 1980). The liver is the most active among the organs in the synthesis of PLP and is the main source for supply of this coenzyme to the blood plasma (Lumeng et al., 1974). Synthesis of PLP may be regulated by PLP itself by inhibiting both pyridoxal kinase and pyridoxamine phosphate oxidase in the liver. The concentration of total PLP in rat liver cytosol is equivalent to that required for inhibition of the purified enzymes (Lui et al., 1981).

Another important difference we observed between liver and brain pyridoxal kinases is the reaction mechanism. The kinetic patterns of product inhibition were consistent with an ordered mechanism for the liver enzyme in contrast to the random mechanism previously reported for the brain enzyme (Churchich & Wu, 1981).

Pyridoxal kinase was inactivated by AP_4-PL and AP_4-PN lacking a reactive formyl group in time-dependent manners. The patterns of the inhibition by AP_4-PL are consistent with the mechanism in which the inhibitor and the enzyme form an E·I complex prior to the formation of an E*·I complex. This mechanism is one of the typical mechanisms for slow-binding inhibition (Morrison & Walsh, 1987; Schloss, 1988). In the pre steady state, the ATP moiety of AP_4-PL occupies the ATP-binding site of the enzyme, whereas its pyridoxal moiety does not occupy the pyridoxal-binding site. Although AP_3-PL contains ATP and pyridoxal moieties, it does not inactivate the enzyme in a time-dependent manner, suggesting that the insertion of one extra phosphate is important for slow-binding inhibition. It should be noted that insertion of one or two extra phosphate moieties besides the structural elements for both substrates can improve the affinities of the resultant bisubstrate analogues for nucleoside, deoxynucleoside, and nucleotide kinases (Bone et al., 1986a,b; Ikeda et al., 1986). For example, adenylate kinase is more strongly inhibited by diadenosine pentaphosphate ($K_i = 2.5 \times 10^{-9}$ M) (Lienhard & Secemski, 1973) than by diadenosine tetraphosphate ($K_i = 2.4 \times 10^{-5}$ M) (Purich & Fromm, 1972).

Byers (1978) suggested that the K_i value of a bisubstrate analogue is lower than the product of the Michaelis constants of each substrate (K_aK_b) if it acts as a transition-state analogue:

$$K_aK_b/K_i \geq 1$$

Inhibition of adenylate kinase by diadenosine pentaphosphate ($K_aK_b = 21$) is this case. However, in inhibition of pyridoxal kinase by AP_4-PL, the $K_aK_b/K_i = 6.7 \times 10^{-4}$. In this sense AP_4-PL is not a transition-state analogue for pyridoxal kinase.

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Registry No. ATP, 56-65-5; AP_4-PL, 101418-64-8; PLP, 54-47-7; pyridoxal, 66-72-8; pyridoxal kinase, 9026-42-0.

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Kinetic Characterization of the [3′-^{32}P]Coenzyme A/Acetyl Coenzyme A Exchange Catalyzed by a Three-Subunit Form of the Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase from *Clostridium thermoaceticum*†

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ABSTRACT: The ability of acetyl coenzyme A synthesizing carbon monoxide dehydrogenase isolated from *Clostridium thermoaceticum* to catalyze the exchange of [3′-^{32}P]coenzyme A with acetyl coenzyme A is studied. This exchange is found to have a rate exceeding that of the acetyl coenzyme A carbonyl exchange also catalyzed by CO dehydrogenase ([1-^{14}C]acetyl coenzyme A + CO ⇌ acetyl coenzyme A + ^{14}CO). These two exchanges are diagnostic of the ability of CO dehydrogenase to synthesize acetyl coenzyme A from a methyl group, coenzyme A, and carbon monoxide. The kinetic parameters for the coenzyme A exchange have been determined: K_m(acetyl coenzyme A) = 1500 μM, K_m(coenzyme A) = 50 μM, and V_{max} = 2.5 μmol min^{-1} mg^{-1}. Propionyl coenzyme A is shown to be a substrate ($K_m \approx 5$ mM) for the coenzyme A exchange, with a rate $^1/_{15}$ that of acetyl coenzyme A, but is not a substrate for the carbonyl exchange. CO dehydrogenase capable of catalyzing both these two exchanges, and the oxidation of CO to CO_2, is isolated as a complex of molecular weight 410 000 consisting of three proteins in an $\alpha_2\beta_2\gamma_2$ stoichiometry. The proposed γ subunit, not previously reported as part of CO dehydrogenase, copurifies with the enzyme and has the same molecular weight on sodium dodecyl sulfate–polyacrylamide gel electrophoresis as the disulfide reductase previously separated from CO dehydrogenase in a final chromatographic step.

Recent work has outlined the biosynthetic pathways by which acetogenic and methanogenic bacteria conduct artful chemistry (including C–C bond assembly) with single-carbon units, enabling these organisms to grow with CO_2 as their sole source of carbon (Wood et al., 1986; Ljungdahl & Wood, 1982; Diekert & Thauer, 1978; Diekert et al., 1979). In acetogenic bacteria, the enzyme responsible for acetyl-CoA[1] synthesis catalyzes the formation of a key carbon–carbon bond between carbon monoxide (enzymically generated from CO_2) and a methyl group (transferred from a corrinoid protein). This extremely oxygen-labile enzyme has been isolated from *Clostridium thermoaceticum* (Drake et al., 1980; Ragsdale et al., 1983) and characterized as containing nickel, iron, and zinc with two subunits of 78 and 71 kDa (Ragsdale et al., 1983). As this protein also belongs to a large class of oxidoreductases known to catalyze the oxidation of CO to CO_2, it has commonly been called carbon monoxide dehydrogenase (CODH), but we have suggested it also be termed an acetyl-CoA synthase to indicate its physiological C–C assembly role.

CO dehydrogenase/acetyl-CoA synthase from *C. thermoaceticum* has been shown by Wood and colleagues to catalyze two diagnostic isotopic exchange reactions, indicative of the unique ability of the enzyme to assemble the acetyl group of acetyl-CoA from one-carbon units (Ragsdale & Wood, 1985; Pezacka & Wood, 1986):

$$CH_3{}^{14}CO\text{—}SCoA + CO \rightleftharpoons CH_3CO\text{—}SCoA + {}^{14}CO \quad (1)$$

$$CH_3CO\text{—}SCoA + {}^*CoASH \rightleftharpoons CH_3CO\text{—}S{}^*CoA + CoASH \quad (2)$$

The exchange of [1-^{14}C]acetyl-CoA with CO (eq 1), first demonstrated by Ragsdale and Wood (1985) (but not well

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[1] Abbreviations: CODH, carbon monoxide dehydrogenase; CoA, coenzyme A; AcCoA or acetyl-CoA, acetyl coenzyme A; PrCoA or propionyl-CoA, propionyl coenzyme A; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EPR, electron paramagnetic resonance; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

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