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Kinetic Evidence for a (4-Amino-2-methyl-5-pyrimidinyl)methyl-Enzyme Intermediate in the Thiaminase I Reaction*

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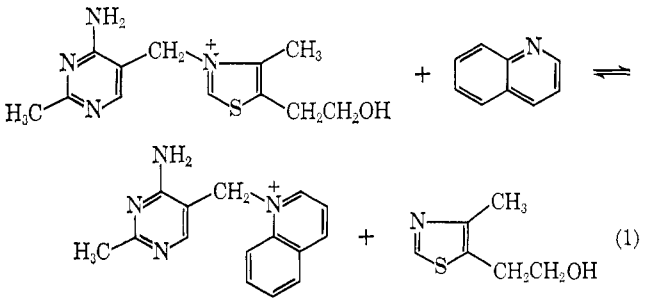
ABSTRACT: Thiaminase I (thiamine:base (4-amino-2-methyl-5-pyrimidinyl)methyl (PM) transferase, EC 2.5.1.2) catalyzes the transfer of the PM group from thiamine and other similar PM-N^+ compounds to aromatic nitrogenous bases and thiols. A kinetic study of this reaction has been made with the bacterial enzyme.

Rates were measured spectrophotometrically or by the incorporation of radioactive label. The following findings suggest that the reaction proceeds by way of a PM-enzyme intermediate. (a) The values of V_{max} for the reaction of 1-PM-quinolinium with pyridine, benzenethiol, and *p*-nitrobenzenethiol are nearly identical. (b) The values of V_{max} for the reaction of quinoline with thiamine, pyrithiamine, and 1-PM-3-chloropyridinium are identical. (c) The observed value of V_{max} for the reaction of 1-PM-pyridinium with quinoline can be calculated from the value of V_{max} for the reaction of 1-PM-pyridinium with *p*-nitrobenzenethiol and the value of V_{max} in b, if the kinetic scheme is assumed to be the Ping-Pong type. (d) The second-order rate constants for the reaction aniline with five PM-N^+ compounds are identical. The following new compounds have been prepared and characterized: 1-PM-quinolinium chloride hydrochloride, 1-PM-pyridinium chloride hydrochloride, 1-PM-3-chloropyridinium chloride hydrochloride, 1-PM-3-methylimidazolium chloride hydrochloride, *S*-PM-benzenethiol, *S*-PM-*p*-nitrobenzenethiol, and [2-^{3}H]quinoline.

Thiaminase I is an enzyme that catalyzes the transfer of the PM group[1] from thiamine and certain PM-N^+ analogs of thiamine to aromatic nitrogenous bases and to thiols (Fujita, 1954; Murata, 1965). Equation 1 shows this reaction with



(1)

thiamine and quinoline. The substrate specificities of thiaminase I's from various sources have been examined in some detail (Fujita, 1954; Murata, 1965), but almost nothing is known about the mechanism of this enzymatic aralkyltransfer reaction. This paper describes a kinetic study of the thiaminase I reaction, the goal of which was to determine whether or not the reaction proceeds by way of a PM-enzyme intermediate. The bacterial enzyme (Ebata and Murata, 1961; Wittliff and Airth, 1968) was used.

Methods

Kinetics. The rates of the reactions catalyzed by thiaminase I were followed spectrophotometrically with a Gilford Model 240 recording spectrophotometer, the cell compartment of which was thermostatted at 25.0 ± 0.1°. Reaction mixtures that contained the buffer and the substrates were prepared in 3.0-ml, 1-cm, Teflon-stoppered cuvets and were temperature equilibrated in a water bath at 25.0° for 15 or more min. Reaction was initiated by the addition of an aliquot from a stock solution of thiaminase I maintained at 25.0°. The recording of the rate was started between 15 and 25 sec after the addition of enzyme and was continued for several minutes, against a blank identical with the reaction mixture except for the absence of enzyme. The total volume of each reaction mixture was 3.0 ml. The buffer present in all the reaction mixtures was 0.1 M sodium phosphate (pH 6.45). The activity of thiaminase I is maximal in this buffer (Wittliff and Airth, 1968). The amount of enzyme present in each reaction mixture was such that no more than 10% of either substrate was consumed during the first minute of reaction. In every case the recording of absorbance against time was linear during the ini-

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[1] The abbreviation PM is used for the (4-amino-2-methyl-5-pyrimidinyl)methyl group. The names, PM-aniline, PM-3-chloropyridinium, PM-pyridinium, PM-*N*-methylimidazolium, and PM-quinolinium refer to the compounds in which the PM group is bonded to the nitrogen of aniline, 3-chloropyridine, pyridine, *N*-methylimidazole, and quinoline, respectively. The names PM-benzenethiol and PM-*p*-nitrobenzenethiol refer to the compounds in which the PM group is bonded to the sulfur of benzenethiol and *p*-nitrobenzenethiol.

tial 30-sec recording. Initial rates, in units of micromolar per minute, were calculated from the slopes of these linear plots by dividing the slopes by the difference between the sum of the micromolar absorptivities of the products and that of the substrates (see Table I). All the initial rates reported in this paper have been normalized to an enzyme concentration of 10 munits/ml by multiplying the unnormalized initial rates by the factor, 10/concentration of enzyme used, in munits/ml.

Thiaminase I was assayed by its catalysis of the reaction of aniline with thiamine, according to the method of Douthit and Airth (1966). The assay mixture contained 0.93 mM aniline, 0.093 mM thiamine, and 0.093 M sodium phosphate buffer (pH 6.45) in a 3-ml volume; the assay was carried out as described above for the kinetic measurements in general. The wavelength for this assay was 248 mμ. At this wavelength and under these conditions the difference between the sum of the molar extinction coefficients of the products, PM-aniline and 5-(2-hydroxyethyl)-4-methylthiazole, and that of the substrates is 11,200. One milliunit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 mμmole of product per min in the assay.

The initial rate in the thiaminase I assay was found to be directly proportional to the concentration of enzyme in the range of 0.5–7 munits/ml. This range encompasses the range used in the kinetic studies. The enzyme was assayed at the beginning and end of each day of rate measurements, and the two assays were always found to be the same within ±5% of the average value.

Radioactivity. The radioactivity of dilute solutions of labeled quinoline and PM-quinolinium was measured with a Packard Model 577 liquid scintillation counter with the scintillator solution described previously (Lienhard and Wang, 1968). When solid PM-quinolinium chloride hydrochloride of low specific activity was counted, it was necessary to use 10% Beckmann Bio-Solv in toluene containing 2,5-diphenyloxazole (15.4 g/gal) as the scintillator solution in order to dissolve the compound. Corrections for quenching were made by the use of [^{3}H]toluene as an internal standard.

Spectroscopic Methods. Proton magnetic resonance spectra were taken in deuterium oxide with about 0.5 M compound on a Varian A-60 spectrometer operating at 60.00 MHz. Each signal has been expressed as follows: δ (apparent multiplicity, relative integrated intensity of the signal, assignment to hydrogen(s) in the compound), where δ is the chemical shift of the signal in parts per million relative to the external standard of tetramethylsilane in chloroform. Ultraviolet spectra were taken with a Cary Model 14 or Unicam SP 800 recording spectrophotometer.

Analyses were done by Scandinavian Microanalytical Laboratory.

Materials

Thiaminase I was partially purified from the culture medium of *Bacillus thiaminolyticus* Matsukawa and Misawa, a strain of which, designated "13023 from R. Kimura," was obtained from the American Type Culture Collection. The purification followed the method of Wittliff and Airth (1968) and was carried out through the Sephadex G-25 chromatography step. This yielded a preparation of enzyme with a specific activity of 400 munits/mg of protein, which was 10 times greater than that in the culture medium. According to the data of Wittliff and Airth (1968), about 7% of the total protein is enzyme at this stage in the purification procedure. The enzyme was stored at −20° in 0.015 M sodium phosphate buffer (pH 6.45). Stock solutions of the enzyme were prepared by thawing small amounts of this enzyme solution and diluting them with 0.015 M sodium phosphate buffer (pH 6.45). These stock solutions were left at room temperature for 16–20 hr before use in the kinetic studies because the enzymatic activity increased about fivefold during the first 16 hr after their preparation. Subsequently, it remained constant for at least 12 hr. The activity of the enzyme which was stored at −20° did not change over the period of 2 years.

Certified ACS pyridine from Fisher Scientific Co., sodium *p*-hydroxymercuribenzoate and PM-N^+H_3 chloride hydrochloride from Sigma Chemical Co., and thiamine chloride hydrochloride from Aldrich Chemical Co. were used without further purification. Commercial preparations of quinoline, aniline, *N*-methylimidazole, benzenethiol, and 3-chloropyridine were purified by distillation. Quinaldic acid and *p*-nitrobenzenethiol were recrystallized from benzene and acetic acid, respectively. Pyrithiamine chloride hydrochloride (1-PM-3-(2-hydroxyethyl)-2-methylpyridine), 5-(2-hydroxyethyl)-4-methylthiazole, PM-chloride dihydrochloride, and 3-(2-hydroxyethyl)-2-methylpyridine were generously given by Dr. Edward Rogers of Merck Sharp and Dohme Research Laboratories. The pyrithiamine and 3-(2-hydroxyethyl)-2-methylpyridine (mp 58–59°, lit. (Raffauf, 1950) mp 55–57°) were used without further purification. The 5-(2-hydroxyethyl)-4-methylthiazole was distilled under vacuum (bp 134° (3.5), lit. (Stein *et al.*, 1941) bp 106–107° (1.2)). The PM-chloride dihydrochloride was converted into the monohydrochloride by crystallization from absolute ethanol. PM-aniline (mp 168–170°, lit. mp 168–170°) was prepared by the condensation of PM-chloride hydrochloride and aniline, after the method of Fujita *et al.* (1952a). The proton magnetic resonance spectrum in 1 N DCl showed 2.60 (singlet, 3 H, 2-CH_3 of pyrimidine), 4.72 (singlet, 2 H, bridge CH_2), 7.63–7.75 (undefined multiplet, 5 H, phenyl hydrogens), and 8.10 (singlet, 1 H, H at C-6 of pyrimidine).

PM-quinolinium chloride hydrochloride was prepared by heating PM-chloride hydrochloride with a fourfold molar excess of quinoline at 100° for 1 hr. The crude product was precipated completely from the cooled reaction mixture by the addition of ether and recrystallized several times from ethanol–water. The yield of crude product was 90%. *Anal.* Calcd for $C_{15}H_{16}Cl_2 \cdot H_2O$: C, 52.80; H, 5.32; Cl, 20.78; N, 16.42. Found: C, 52.28; H, 5.67; Cl, 20.71; N, 16.23. When a sample of the compound was stored in an evacuated desiccator over P_2O_5 for 2 and 5 days, its weight decreased to 94.3% of its original value (calculated per cent for loss of 1 mole of H_2O, 94.72%). When this sample was subsequently left open to the air for 1 day, its weight was again the original weight and remained so. The proton magnetic resonance spectrum in D_2O showed 2.55 (singlet, 3 H, 2-CH_3 of pyrimidine), 6.16 (singlet, 2 H, bridge CH_2), 7.66 (singlet, 1 H, H at C-6 of pyrimidine) and 7.90–8.53 and 9.10–9.40 (undefined multiplets, 7 H, hydrogens of quinolinium nucleus). The assignment of the signal at 7.66 to the hydrogen at C-6 of the pyrimidine is based upon comparison with the spectrum of *N*-methylquinolinium chloride (prepared by the procedure of Osborne *et al.* (1956)), which showed the two multiplets but not the singlet at 7.66.

TABLE I: pK's, Molecular Weights, and Ultraviolet Spectra of Compounds Used.[a]

Compound	pK	Mol Wt	Calcd Mol Wt	λ_{max} (log ϵ)	λ (log ϵ)[b]
PM-aniline (**1**)				240 (4.270)	236 (4.246), 248 (4.188) 251 (4.131), 319 (2.48)
Thiamine chloride hydrochloride (**2**)	4.68	341[c]	337.3	233 (4.052), 268 (3.935)	236 (4.041), 248 (3.832), 319 (<2)
PM-3-chloropyridinium (**3**) chloride hydrochloride	4.62	308	308.6	231 (4.100), 275 (3.964)	248 (3.648), 319 (<2)
PM-quinolinium chloride hydrochloride·H_2O (**4**)	4.87	348	341.2	236 (4.646), 278 (3.825), 318 (3.935)	251 (3.707), 319 (3.932), 335 (3.574)
PM-pyridinium chloride hydrochloride·$2H_2O$ (**5**)	4.73	308	309.2	235 (4.042), 260 (3.857)	248 (3.800), 319 (<2)
Pyrithiamine chloride hydrochloride (**6**)	4.78	336[c]	331.3	233 (4.060), 272 (4.072)	248 (3.793), 319 (<2)
PM-N-methylimidazolium chloride hydrochloride (**7**)	4.93	283[c]	276.3	232 (4.013), 274 (3.700)	248 (3.624)
PM-ammonium chloride hydrochloride (**8**)	5.0, 8.2[d]	220	209.1		248 (3.613)
PM-benzenethiol (**9**)				242 (4.061)	319 (<2)
PM-*p*-nitrobenzenethiol (**10**)				238 (4.140), 343 (3.978)	330 (3.954), 410 (2.70)
Aniline (**11**)	4.6[e]			230 (3.903), 280 (3.100)	236 (3.833), 248 (3.191) 251 (3.00), 319 (<2)
5-(2-Hydroxyethyl)-4-methylthiazole (**12**)	3.5[f]			250 (3.623)	236 (3.380), 248 (3.626), 319 (<2)
3-Chloropyridine (**13**)	2.8[g]			267 (3.489)	248 (3.057), 319 (<2)
Quinoline (**14**)	4.9[g]			313 (3.592), 299, 288, 277, 230, 225 (4.534)	236 (3.624), 251 (3.218), 319 (2.653)
Pyridine (**15**)	5.2[g]				248 (3.352), 319 (<2)
3-(2-Hydroxyethyl)-2-methylpyridine (**16**)	6.3[h]			266 (3.760)	248 (3.210)
N-Methylimidazole (**17**)	7.3[g]				248 (<2)
Benzenethiol (**18**)	6.5[i]				319 (<2)
p-Nitrobenzenethiol (**19**)	5.1[j]			410 (4.152)	330 (3.326), 410 (4.152), 440 (4.008), 470 (3.638)

[a] Unless stated otherwise, the pK's and molecular weights were determined by titration to pH 8 of about 0.01 M solutions with sodium hydroxide at room temperature. All spectra were taken in 0.1 M sodium phosphate buffer (pH 6.45), against a blank of this buffer. [b] These λ are ones at which kinetic measurements were made. [c] Of sample stored in an evacuated desiccator over P_2O_5. [d] By titration to pH 11. [e] At 30°, Brown *et al.* (1955). [f] From a spectrophotometric titration at 270 mμ in hydrochloric and acetic acid buffers, at 0.1 M ionic strength and 25°. [g] At 25°, Albert (1963). [h] From a spectrophotometric titration at 270 mμ in sodium phosphate buffers at 0.1 M ionic strength and 25°. [i] At 25°, Kreevoy *et al.* (1960). [j] Ellman (1958).

PM-pyridinium chloride hydrochloride was made by refluxing PM-chloride hydrochloride with a 12-fold molar excess of pyridine. Removal of the pyridine on the rotary evaporator gave crude yellow product in 80% yield. It was decolorized by treatment of an ethanol–water solution with charcoal at room temperature. The solvent was then removed removed on the rotary evaporator, and the colorless powder was recrystallized from ethanol–water. *Anal.* Calcd for $C_{11}H_{14}Cl_2N_4 \cdot 2H_2O$: C, 42.73; H, 5.87; Cl, 22.93; N, 18.12. Found: C, 42.50; H, 5.93; Cl, 23.11; N, 18.35. When a sample of the compound was stored in an evacuated desiccator over P_2O_5 for 1 and for 4 days, its weight decreased to 88.4% of the original value (calculated for the loss of 2 moles of water, 88.36%). Upon storage in air for 1 day this sample returned to its original weight. The proton magnetic resonance spectrum in 0.1 N DCl showed 2.63 (singlet, 3 H, 2-CH_3 of pyrimidine), 5.88 (singlet, 2 H, bridge CH_2), and 8.00–9.00 (undefined multiplet and probably a singlet at 8.20 due to the H at C-6 of the pyrimidine, 6 H, all the aromatic hydrogen atoms).

The synthesis of PM-3-chloropyridinium chloride hydrochloride was carried out in a way similar to that of PM-pyridinium chloride hydrochloride. *Anal.* Calcd for $C_{11}H_{13}Cl_3N_4$: C, 42.81; H, 4.57; Cl, 34.46; N, 18.15. Found: C, 42.54; H, 4.36; Cl, 34.31; N, 18.19. The proton magnetic resonance spectrum showed 2.60 (singlet, 3 H, 2-CH_3 of pyrimidine), 5.86 (singlet, 2 H, bridge CH_2), and 7.9–9.1 (undefined multiplets and probably a singlet at 8.25 due to H at C-6 of the pyrimidine, 5 H, all the aromatic hydrogen atoms).

PM-*N*-methylimidazolium chloride hydrochloride was syn-

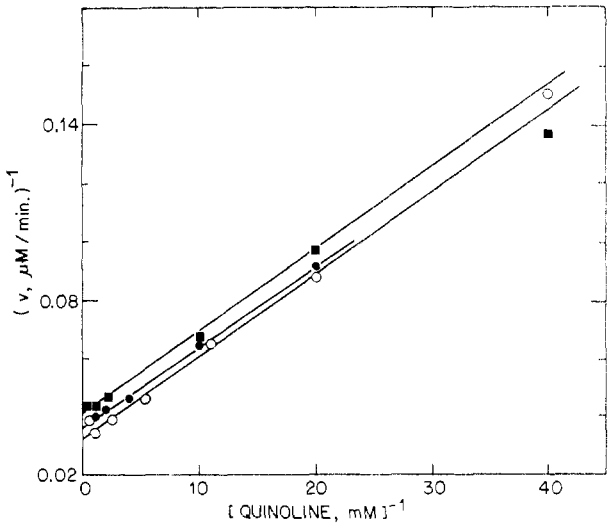


FIGURE 1: Lineweaver–Burk plots of the kinetic data for the reaction of thiamine with quinoline, catalyzed by thiaminase I. The single concentrations of thiamine with each set of concentrations of quinoline were 33 (■), 100 (●), and 300 (○) μM.

thesized by reacting PM-chloride hydrochloride with a fourfold molar excess of *N*-methylimidazole for 1 hr at 50°. The reaction mixture was diluted with water, adjusted to pH 10 with 1 N NaOH, extracted with ether, and taken to dryness on the flash evaporator. The residue was extracted with ethanol, and the product chloride was precipitated from the ethanolic extract by the addition of ether. Treatment with ethanolic HCl yielded the chloride hydrochloride, which was recrystallized from ethanol–water. *Anal.* Calcd for $C_{10}H_{15}Cl_2N_5 \cdot 0.33H_2O$: C, 42.56; H, 5.60; Cl, 25.13; N, 24.82. Found: C, 42.80; H, 5.65; Cl, 24.97; N, 24.52. The proton magnetic resonance spectrum showed 2.62 (singlet, 3 H, 2-CH_3 of pyrimidine), 3.92 (singlet, 3 H, N-CH_3), 5.42 (singlet, 2 H, bridge CH_2), 7.54 (undefined multiplet, 2 H, hydrogen atoms at C-4 and -5 of imidazole), 8.13 (singlet, 1 H, hydrogen atom at C-6 of pyrimidine), and 8.86 (undefined multiplet, 1 H, hydrogen atom at C-2 of imidazole.

PM-*p*-nitrobenzenethiol was prepared by adding 5 ml of 2 M aqueous PM-chloride hydrochloride to a solution of 10 mmoles of *p*-nitrobenzenethiol in 20 ml of 1 N KOH, with stirring and in an atmosphere of argon. The insoluble product was collected, washed with water, and dissolved in 250 ml of 0.04 N HCl. The acidic solution was filtered, and the filtrate was neutralized with 1 N KOH. Crude product precipitated in 60% yield. After crystallization from ethanol, its melting point was 212.5–215°. *Anal.* Calcd for $C_{12}H_{12}N_4O_2S$: C, 52.16; H, 4.38; N, 20.28; S, 11.60. Found: C, 52.06; H, 4.45; N, 20.19; S, 11.73.

The preparation of PM-benzenethiol was similar to that of PM-*p*-nitrobenzenethiol. The melting point after crystallization from carbon tetrachloride was 146.5–147.5°. *Anal.* Calcd for $C_{12}H_{13}N_3S$: C, 62.31; H, 5.66; N, 18.17; S, 13.86. Found: C, 61.64; H, 5.55; N, 17.77; S, 13.72.

Table I summarized the p*K*'s, molecular weights, and ultraviolet spectra of the compounds used in this study.

[2-^{3}H]Quinoline hydrochloride was prepared by the decarboxylation of quinaldic acid (Brown and Hammick, 1949) in bis(2-ethoxyethyl) ether containing [^{3}H]H_2O. Quinaldic acid (2 mmoles) was dissolved in 5 ml of bis(2-ethoxyethyl) ether which had been dried by passage through aluminum oxide; 10 μl of [^{3}H]H_2O (1 Ci/ml) was added, and the mixture was heated at 183° for 1 hr. The reaction mixture was cooled and acidified with 0.75 ml of 2.9 N ethanolic HCl. The quinoline hydrochloride was then precipitated by the addition of 100 ml of anhydrous ether. Exchangeable tritium was removed from the product by dissolving it in 10 ml of ethanol and removing the ethanol with a flash evaporator, six times. The deliquescent hydrochloride (final yield, 1 mmole) was stored in an evacuated desiccator over P_2O_5. The compound was used without further purification. It was pure according to the following criteria: (a) its ultraviolet spectrum was identical with that of authentic quinoline hydrochloride; (b) the specific activity of a sample was the same before and after ion-exchange chromatography on Bio-Rad AG 50W-X8 H^+ form; (c) the initial rates of reaction of 2-[^{3}H]quinoline and of authentic quinoline with thiamine, catalyzed by thiaminase I and measured at 319 mμ, were identical.

Results

Rates of Reaction of PM Compounds with Nitrogen Heterocycles and Thiols. If the thiaminase I reaction proceeds by way of a (4-amino-2-methyl-5-pyrimidinyl)methyl-enzyme intermediate (see the Discussion), its kinetics would probably be described by the Ping-Pong kinetic scheme (eq 2), where S_1 is

$$\begin{aligned} E + S_1 &\underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow{k_2} E' + P_1 \\ E' + S_2 &\underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} E'S_2 \xrightarrow{k_2'} E + P_2 \end{aligned} \tag{2}$$

the reactant PM compound, S_2 is the reactant nucleophile, P_1 is the product nucleophile, P_2 is the product PM compound, and E′ is the PM-enzyme intermediate. This scheme yields the following equation for the dependence of the initial rates upon the concentration of the substrates under steady-state conditions

$$v = \frac{\left(\frac{k_2 k_2'}{k_2 + k_2'}\right)[E]_{total}[S_1][S_2]}{\left(\frac{k_2}{k_2 + k_2'}\right)\left(\frac{k_{-1}' + k_2'}{k_1'}\right)[S_1] + \left(\frac{k_2'}{k_2 + k_2'}\right)\left(\frac{k_{-1} + k_2}{k_1}\right)[S_2] + [S_1][S_2]} \tag{3}$$

The Lineweaver–Burk transformation of eq 3 shows that a series of plots of $1/v$ *vs.* $1/[S_2]$ (or $1/[S_1]$) for constant values of $[S_1]$ (or $[S_2]$) should be parallel straight lines. This pattern contrasts with the curved lines or converging straight lines that are predicted by kinetic schemes for two-substrate enzymic reactions which proceed *via* a ternary complex (ES_1S_2) (Cleland, 1963; Florini and Vestling, 1957).

Figure 1 presents Lineweaver–Burk plots for the reaction of thiamine at constant concentrations of 33, 100, and 300 μM with quinoline, catalyzed by thiaminase I. The lines have purposely been drawn as parallel in order to show that the kinetic data is not inconsistent with the Ping-Pong scheme. However,

TABLE II: V_{max} and K_m Values for the Reaction of PM Compounds with Nucleophiles.[a]

PM Compound	Concn (μM)	Nucleophile	Concn Range (μM)	λ (mμ)[b]	K_m (μM)[c]	V_{max} (μM/min)	K_m^∞ (μM)[d]	V_{max}^∞ (μM/min)[e]
Thiamine (**1**)	33	Quinoline	25–2,000	236	67	24.2	83	30.5
	33		25–2,000	319	62	23.5		
	100		25–2,000	319	79	28.4		
	300		25–2,000	319	80	30.0		
PM-3-chloropyridinium (**2**)	300	Quinoline	25–2,000	319	80	29.5	82	29.2
	1000		25–2,000	319	84	29.0		
PM-quinolinium[f] (**3**)		Quinoline						21 ± 4
PM-pyridinium[g] (**4**)	300	Quinoline	25–2,000	319	35	9.2	55	14.3
	1000		25–2,000	319	47	12.3		
Pyrithiamine (**5**)	33	Quinoline	25–2,000	319	60	22.4	75	29.0
	100		25–2,000	319	62	25.7		
	300		25–2,000	319	80	28.2		
PM-pyridinium (**6**)	100	*p*-Nitrobenzenethiol[h]	20–150	410	<10	13.5	<10	24.0
	250		23–93	410	<10	18.8		
	250		25–200	330	<10	21.0		
	500		16–125	410	<10	19.9		
	2500		23–93	410	<10	23.6		
PM-quinolinium[i] (**7**)	300	Benzenethiol[h]	64–1,930	319	170	192	150	191
	600		80–2,350	335	130	190		
PM-quinolinium[i] (**8**)	300	*p*-Nitrobenzenethiol[h,j]	17–350	470–410	~17	180	~17	180
	600		17–350	470–410	~17	180		
PM-quinolinium[i] (**9**)	300	Pyridine	250–10,000	319	670	174	690	174
	600		250–10,000	335	710	174		
	300[k]		100–10,000	319	650	146		
PM-quinolinium[i] (**10**)	300	5-(2-Hydroxyethyl)-	250–20,000	319	560	110	600	111
	600	4-methylthiazole	250–20,000	335	630	111		
	300[k]		50–10,000	319	620	120		
PM-quinolinium[i] (**11**)	300	2-Chloropyridine	750–29,000	319	3100	79	3500	78
	600		750–29,000	335	3800	77		

[a] Each determination of K_m and V_{max} was made by measuring the initial rates at the stated concentration of the PM compound and 4 to 7 concentrations of the nucleophile in the stated concentration range. V_{max} and K_m were taken as the ordinate intercept and slope, respectively, of the plot of initial rate against initial rate/[nucleophile]. [b] At which rates were measured. [c] Of nucleophile. [d] Ordinate intercept of the plot, K_m *vs.* K_m/[PM compound]. [e] Ordinate intercept of the plot, V_{max} *vs.* V_{max}/[PM compound]. [f] See Table III and the text. [g] The rates of reaction of 100 μM PM-pyridinium with quinoline were measured, but accurate initial rates were not obtained because of a marked deceleration during the first minute of reaction. The cause is apparently inhibition by PM-quinolinium; the initial rate of reaction between 100 μM PM-pyridinium and 900 μM quinoline in the presence of 4 μM PM-quinolinium is only 64% of the approximate initial rate in its absence. [h] Stock solutions of the thiols in deoxygenated water were maintained at 25° and added to the reaction mixture, less enzyme, within the minute before the initiation with enzyme. [i] Each reaction mixture contained 0.2 mg/ml of crystalline bovine serum albumin from Sigma Chemical Co. [j] In this case only blanks which contained all the components of the reaction mixture except the PM compound showed measurable rates, probably due to the oxidation of the thiol to the disulfide. Each blank rate was no more than 10% of the rate with the corresponding complete reaction mixture. Correction was made by subtraction of the blank rate. [k] Bovine serum albumin was omitted from the reaction mixtures.

the combination of small deviations from the straight line within each set with the close grouping of the lines makes it apparent that the data do not require parallel lines. Thus, these data are ambiguous. It probably would have been possible to decide whether the data described parallel lines if sets of rate measurements had been made at constant concentrations of thiamine at and below its K_m, since the plots for the different constant concentrations of thiamine would then have been more separated. However, these measurements were not made because the spectrophotometric method is not sensitive enough to yield accurate initial rates for this reaction when the concentration of thiamine is lower than about 20 μM. In fact, the insufficient sensitivity of the spectrophotometric method prevented a definitive kinetic analysis of this type for any of the combinations of PM compounds and nucleophiles which are described below.

TABLE III: Exchange Reaction between PM-quinolinium and [2-^{3}H]Quinoline.[a]

PM-quinolinium (mM)	[2-^{3}H]-Quinoline (mM)	Enzyme (munits/ml)	Time[b] (sec)	cpm Incorpd[c]	Normalized Rate[d] (μM/min)
0.17	1.0	1.87	25	1440	
			85	3780	22.8
			145	5470	16.6[e]
			205	7750	22.1[f]
0.17	2.0	2.68	25	1890	
			85	4680	19.0
			25	1670	
			85	4900	21.8
0.10	1.0	3.00	25	2010	
			85	5320	20.0
			25	1940	
			85	6050	25.0
0.10	2.0	3.00	25	1920	
			85	5400	21.0
			25	1890	
			85	5180	19.9

[a] Each reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.45), [2-^{3}H]quinoline (specific activity 550,000 cpm/μmole), PM-quinolinium, and thiaminase I. The reaction mixture less the enzyme was prepared in a stoppered test tube and maintained at 25° for at least 15 min. Reaction was initiated by addition of the enzyme from a stock solution at 25°. After 15 sec, 2.0 ml of the reaction mixture was withdrawn with a blow-out pipet, and after 25 sec the contents of the pipet were rapidly mixed with 0.20 ml of 5 mM *p*-hydroxymercuribenzoate. This procedure was repeated at 1-min intervals. Unreacted [2-^{3}H]quinoline was removed from these samples by extracting each sample eight times with 3 ml of water-saturated ether. Aliquots (0.20 ml) of the final ether extracts were counted and, in all cases, contained less than 5 cpm. In order to determine the specific activity of the PM-quinolinium in each sample, an aliquot of the ether-extracted sample was counted and the absorbance at 318 mμ of the sample was measured. These values were then corrected by the subtraction of values obtained for a sample from a blank reaction mixture. The blank reaction mixture was identical with the complete reaction mixture except for the absence of PM-quinolinium and was worked up in the same way. The blank values for counts per minute and absorbance were in all cases less than 30 and 10%, respectively, of the values for the samples from the complete reaction mixtures. The concentration of PM-quinolinium in each sample was calculated from the corrected absorbance at 318 mμ and its ϵ at this wavelength and was about 85% of the concentration in the reaction mixture; the loss is probably due to the extraction of a small amount into the ether. The cpm incorporated into PM-quinolinium in each reaction mixture at each time were calculated from its specific activity and its concentration in the reaction mixture. [b] After addition of the enzyme. [c] Incorporated into the PM-quinolinium in 1 ml of reaction mixture. [d] Normalized rate = 10/[enzyme] $\times$ 1/cpm per mμmole of quinoline $\times$ (cpm incorpd after 85 sec $-$ cpm incorpd after 25 sec). [e] For the period, 85–145 sec. [f] For the period, 145–205 sec.

Table II summarizes the kinetic data for the reactions between a number of PM compounds and nucleophiles, catalyzed by thiaminase I. The values of V_{max} and of K_m for each set were taken from plots of the initial rate (v) *vs.* v/[nucleophile]; this method of plotting the data yields more accurate values than does the Lineweaver–Burk method (Dowd and Riggs, 1965). The plots were approximately linear (the Lineweaver–Burk plots in Figure 1 indicate the amount of scatter); and the values of V_{max} and of K_m are probably within 10 and 20%, respectively, of the true values. At the concentrations of each PM compound which were used, V_{max} and K_m were almost independent of the concentration. Values of V_{max} and K_m for saturating concentrations of the PM compounds (V_{max}^{∞} and K_m^{∞}) were obtained by very short extrapolations based upon the relationships

$$V_{max} = \frac{V_{max}^{\infty}[S_1]}{\text{constant} + [S_1]} \quad (4)$$

and

$$K_m = \frac{K_m^{\infty}[S_1]}{\text{constant} + [S_1]} \quad (5)$$

where $[S_1]$ is the concentration of the PM compound. The equation for V_{max}^{∞} is valid regardless of whether the correct kinetic scheme is the Ping-Pong scheme (see eq 3) or a ternary complex scheme which predicts linear Lineweaver–Burk plots, whereas that for K_m is valid only for the Ping–Pong kinetic scheme (Cleland, 1963; Florini and Vestling, 1957).

Convenient measurement of the rates of reaction of PM-quinolinium with the reactive nucleophiles (Table II, **7–11**) necessitated the use of rather low concentrations of the enzyme (about 0.7 munit/ml). At these concentrations, with some of the nucleophiles, partial inactivation of the enzyme occurred during the first several minutes of reaction. This made it difficult to measure initial rates accurately. It was found that bovine serum albumin at 0.2 mg/ml prevented the inactivation, and consequently these rates were measured in the presence of bovine serum albumin. There was no significant rate of reaction in the presence of albumin in the absence of the nucleophile. Some determinations of the initial rates in the presence and absence of albumin were made for the reactions of PM-quinolinium with various concentrations of benzenethiol, *p*-nitrobenzenethiol, pyridine (Table II, **9**), and 5-(2-hydroxyethyl)-4-methylthiazole (Table II, **10**). In each instance, the rate in the absence of albumin did not differ by more than 16% from the rate in its presence. Thus, the values of V_{max} obtained with albumin present (Table II, **7–11**) can be compared with the values of V_{max} obtained in its absence (Table II, **1–6**) without making any correction for stimulation or inhibition by albumin.

The rates of reaction of PM-quinolinium with quinoline were determined by measuring the formation of labeled PM-quinolinium from [2-^{3}H]quinoline (Table III). The reaction was stopped after various periods by inactivating thiaminase I with 0.5 mM *p*-hydroxymercuribenzoate (Ebata and Murata, 1961). Control experiments in which the reaction of quinoline with thiamine was followed spectrophotometrically at 319 mμ showed that the introduction of this concentration of *p*-hydroxymercuribenzoate completely halted this reaction

within 20 sec. The results in the first experiment of Table III show that the rate of the exchange reaction is approximately constant during the first 0.5–3.5 min. A plot of these data as cpm incorporated into PM-quinolinium against time indicates that about 300 cpm was incorporated at zero time. This apparent burst is probably due to the lack of instantaneous inactivation by the *p*-hydroxymercuribenzoate and also to error in the moment taken as the time of initiation of the reaction. These factors have been eliminated in calculating the rates of the exchange reaction by basing the calculation of rates upon the *differences* in counts per minute incorporated between two times of inactivation. Also, since the amount of incorporation of label into the PM-quinolinium was in all cases 12% or less of the amount expected at isotopic equilibrium, the exchange rates given in Table III are the initial rates for the reaction of quinoline with PM-quinolinium and do not need to be corrected for the exchange of label out of PM-quinolinium (Jencks, 1969). The data in Table III show that the rate of the exchange reaction is not increased when the concentrations of both substrates are doubled. Consequently, this rate is V_{max}^{co} for the reaction of PM-quinolinium.

Rates of Reaction of PM Compounds with Aniline. Figure 2 shows the dependence of the initial rates of the reaction between thiamine and aniline upon the concentrations of the substrates. The rates are independent of the concentration of thiamine in the 33–100-μM range, but are, within experimental error, directly proportional to the concentration of aniline over the 0–1000-μM range. Consequently, these concentrations of thiamine are sufficient for saturation, whereas the concentrations of aniline are less than the K_m for aniline. The large background absorbance at 248 mμ due to aniline (Table I) prevented the measurement of rates at aniline concentrations greater than 1 mM. Very similar kinetic data were obtained for the other reactive PM compounds; the results are summarized in Table IV.

Products of the Thiaminase I Reactions. The discussion to this point has assumed that the predominant reaction catalyzed by thiaminase I is the transfer of the PM group from the PM compound to the added nucleophile. This assumption is supported by all the previous work on the enzyme (see beginning of article), and the products of each reaction have not been isolated and characterized. However, a few experiments which identify some products were carried out. (a) The rates of reaction of thiamine with quinoline (Table II, 1), PM-pyridinium with *p*-nitrobenzenethiol (Table II, 6), and PM-quinolinium with aniline (Table IV, 3) have been measured at more than one wavelength, and the values found at the different wavelengths are the same. Since the calculation of these rates uses the difference between the sum of the micromolar absorptivities of the products and that of the reactants, these identities show that the spectral changes are the ones which accompany formation of the expected products. (b) The thiaminase I catalyzed reaction of 3 mM tritiated quinoline with 0.17 mM PM-quinolinium in 0.1 M sodium phosphate buffer (pH 6.46) was allowed to proceed to 5% of isotopic equilibrium. The reaction was stopped by maintaining the reaction mixture at 100° for 5 min. The specific activity of the PM-quinolinium was determined by the method described in Table III. This method assumes that, after correction for a blank, the radioactivity which cannot be extracted from the aqueous reaction mixture with ether is located in the PM-quinolinium. As a test of this assumption, the ether-extracted reaction mixture was taken to dryness on the flash evaporator and then the PM-quinolinium in the residue was extracted with ethanol. The spectrum of the ethanolic extract was the same as that of authentic PM-quinolinium in ethanol and yielded the concentration of PM-quinolinium. The total cpm in the PM-quinolinium in the ethanolic solution was calculated from this concentration and the specific activity described above. Enough solid PM-quinolinium chloride hydrochloride was suspended in the ethanolic solution so that the specific activity of the compound would be 10.3 cpm/μmole if the above assumption were correct. Water was added to the boiling ethanolic solution to bring all the PM-quinolinium chloride hydrochloride into solution; the compound crystallized upon cooling. Its specific activity was found to be 10.7 cpm/μmole. After recrystallization from ethanol–water the specific activity was 10.6 cpm/μmole.

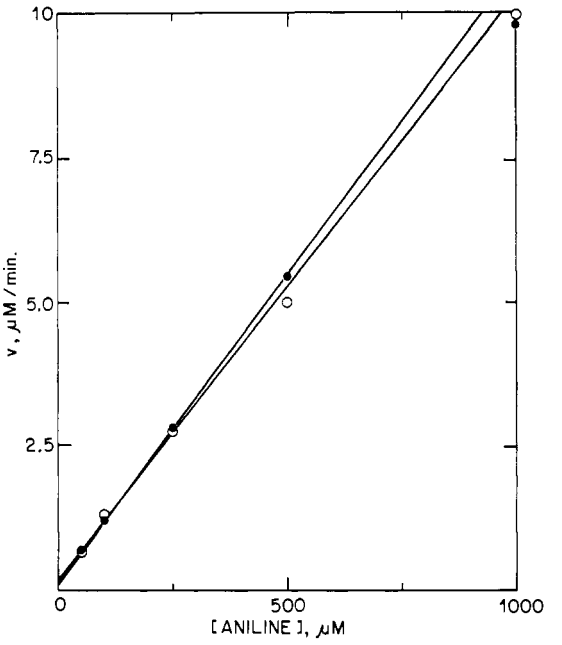


FIGURE 2: Dependence of the rate of the thiaminase I catalyzed reaction between thiamine and aniline upon substrate concentrations. (○) 33 μM thiamine; (●) 100 μM thaimine. The reaction was followed at 248 mμ. The values of v given here are corrected ones which were calculated after subtracting the small rate of change in absorbance which occurs in the absence of aniline from that which occurs in its presence.[2]

Discussion

On the basis of our knowledge of other enzymatic group transfer reactions, there are, *a priori*, two possibilities for the reaction pathway of the thiaminase I reaction. One is reaction *via* a ternary complex of the enzyme, PM compound, and nu-

[2] Small changes in absorbance with time in the absence of another nucleophile were observed at 248 mμ with all the reactive PM compounds. These are probably due to a reaction in which either the four amino group or one of the pyrimidine ring nitrogen atoms of the PM compound acts as the acceptor for the PM group of another substrate molecule, since it is known that both PM-N^+H_3 and PM-OH accelerate the disappearance of thiamine in the presence of crude bacterial thiaminase I (Fujita *et al.*, 1952b).

TABLE IV: Rate Constants for the Reaction of PM Compounds with Aniline, Catalyzed by Thiaminase I.[a]

Substrate	Concn (μM)	Concn Range[a] (mM)	No. of Concn	λ[b]	Rate Constant[c] (μM/min mM)
Thiamine (**1**)	100	0–1	5	248	10.7
	33	0–1	5	248	10.5
PM-3-chloropyridinium (**2**)	200	0–1	5	248	11.6
	100	0–1	5	248	12.0
PM-quinolinium (**3**)	33	0–0.18	3	236	11.1
	100	0–0.50	4	251	11.2
	33	0–0.50	4	251	11.2
	100	0–1	5	319	11.0
	33	0–1	5	319	11.2
PM-pyridinium (**4**)	300	0–0.30	2	248	10.3
	200	0–0.30	2	248	9.3
	100	0–0.30	2	248	9.5
Pyrithiamine (**5**)	100	0–1	5	248	10.3
	33	0–1	5	248	10.3
PM-*N*-methylimidazolium (**6**)	500	0.5	1	248	0[d]
PM-NH_3^+ (**7**)	200	1.0	1	248	0[e]

[a] In this range of concentrations of aniline the corrected initial rates (see legend of Figure 2) are, within experimental error, directly proportional to the concentration of aniline. [b] At which the kinetic measurements were made. [c] Each rate constant (final column) is the slope of the linear plot of the corrected initial rates (see legend of Figure 2) against the millimolar concentration of aniline. [d] No reaction was observed in the presence of 10 munits/ml of thiaminase I. [e] No reaction was observed in the presence of 4 munits/ml of thiaminase I.

cleophile; the other is transfer of the PM group from the PM compound to the enzyme with release of its nitrogenous base, followed by reaction of the PM-enzyme with the acceptor nucleophile (Ping-Pong scheme, see the Results section). The results of this study strongly support the second possibility, for the following reasons. (a) The values of V_{max}^{∞} for the reactions of PM-quinolinium with pyridine, *p*-nitrobenzenethiol, and benzenethiol do not differ from each other by more than 5% of their average value and appear to approach a limiting value of about 190 μM/min, which is greater than that for the reaction of any of the other nucleophiles with PM-quinolinium. These three nearly identical values for V_{max}^{∞} can be explained by an identical reaction, the formation of the PM-enzyme intermediate from enzyme-bound PM-quinolinium, which is entirely or almost entirely rate determining. If the reaction occurred *via* the ternary complex pathway, and the V_{max}^{∞} values were the rates for direct nucleophilic displacement of quinoline, the values of V_{max}^{∞} with the three different nucleophiles would not be expected to be nearly identical. For example, in methanol at 25° the relative rates of direct nucleophilic displacement of iodide ion from methyl iodide by pyridine, benzenethiol, *p*-nitrobenzenethiolate anion, and benzenethiolate anion are $1:2.9:2.9 \times 10^3:4.9 \times 10^4$, respectively (Pearson *et al.*, 1968; Alexander *et al.*, 1968). The relative reactivities of the nucleophiles in complex with thiaminase toward the PM-methylene carbon would undoubtedly be somewhat different, but it seems improbable that these changes would result in nearly identical values of V_{max}^{∞}. (b) The values of V_{max}^{∞} for the reaction of quinoline with thiamine, PM-3-chloropyridinium, and pyrithiamine are identical and larger than the values for the corresponding reaction with PM-quinolinium and PM-pyridinium. This limiting value of V_{max}^{∞} can be explained by an identical rate-limiting reaction, the transfer of the PM group from the PM-enzyme intermediate to enzyme-bound quinoline. If values of V_{max}^{∞} were the rates for direct nucleophilic displacement by quinoline at the methylene carbon of the PM compounds, the V_{max}^{∞} with thiamine, PM-3-chloropyridinium, and pyrithiamine would be expected to differ markedly. This is so because it is known that within a series of compounds with the same type of leaving group the rate of direct nucleophilic substitution (S_N2) reaction with a single nucleophile is inversely dependent upon the pK_a of the leaving group (Streitweiser, 1956). For example, in the reaction $RCOOCH_3 + N(CH_3)_3 \rightarrow RCOO^- + N^+(CH_3)_4$, the methyl ester of an acid which is 100 times stronger reacts 22 times more rapidly (Hammett and Pfluger, 1933). Since the relative basicities of 3-chloropyridine, 5-(2-hydroxyethyl)-4-methylthiazole, and 3-(2-hydroxyethyl)-2-methylpyridine are $1:10^{0.7}:10^{3.5}$ (Table I), the order of reactivity in nonenzymatic S_N2 reactions would almost certainly be PM-3-chloropyridinium > thiamine $\gg$ pyrithiamine. (c) Since the value of V_{max}^{∞} for the reaction of PM-pyridinium with *p*-nitrobenzenethiol (24 μM/min) is less than the limiting value of V_{max}^{∞} described in (a) (190 μM/min), it seems likely that the rate-limiting step in this reaction is also the formation of the PM-enzyme intermediate. According to the Ping-Pong scheme

$$V_{max}^{\infty} = \frac{k_2 k_2'[E]_{total}}{k_2 + k_2'} \quad (6)$$

where k_2 is the first-order rate constant for the formation of the PM-enzyme intermediate and k_2' is the first-order rate constant for the reaction of the intermediate with the acceptor

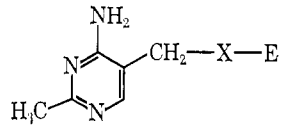
nucleophile (see eq 2). On the basis of the argument presented in (b), the value of $k_2'[E]_{total}$ for quinoline is 29.5 μM/min. Thus, the assumption of the Ping-Pong scheme and these assignments of rate-limiting steps leads to the prediction that the value of V_{max}^{∞} for the reaction of PM-pyridinium with quinoline is (24)(29.5)/(24 + 29.5) = 13.0 μM/min. This value agrees adequately with the experimental value of 14.3 μM/min. In a similar way, the value of V_{max}^{∞} for the reaction of PM-quinolinium with quinoline can be predicted from the limiting values described in (a) and (b) to be 25.5 μM/min, which is in fair agreement with the experimental values of 21 $\pm$ 4 μM/min. (d) The second-order rate constants for the reaction of aniline with five PM-N^+ compounds that vary over a range of $10^{+3.5}$ in the basicities of the leaving nitrogenous base are identical. For the reason stated in (b), this result suggests an identical rate-determining step, the reaction of aniline with a PM-enzyme intermediate.

An alternate interpretation of the identical values of V_{max}^{∞} which have been found for the reaction of quinoline with the three PM compounds is that the rate-determining step in these reactions is the dissociation of the product PM-quinolinium from its complex with thiaminase I. This could be the case for either a Ping-Pong scheme (eq 2) expanded to include enzyme–product complexes or an ordered ternary complex scheme (eq 7 with P_2 = PM-quinolinium). Similarly, the single value

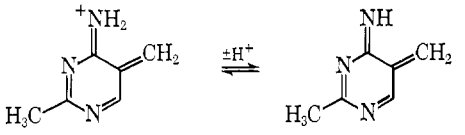
$$E + S_1 \rightleftharpoons ES_1 \overset{\pm S_2}{\rightleftharpoons} ES_1S_2 \xrightarrow{k_t} EP_2 + P_1 \xrightarrow{k_d} E + P_1 + P_2 \quad (7)$$

for the second-order rate constants for the reaction of aniline with the five PM compounds can be explained by an ordered ternary complex scheme in which the dissociation of PM-aniline from thiaminase I is rate limiting, if it is assumed that the $K_{m,app}$ for aniline does not vary with the leaving group of the PM substrate. Also, since the rates with aniline as the nucleophile were determined with concentrations of aniline such that the rate was directly proportional to the concentration of aniline, another possibility is that the rate-limiting step under these conditions is the binding of aniline. Again, this could be the case for either a Ping-Pong scheme in which $k_2' \gg k_{-1}'$ and so $v = k_1'[E]_{total}$[aniline] (see eq 3), or an ordered ternary complex scheme (eq 7, with S_1 = aniline and S_2 = the PM compound). While no decision among these possibilities can be made from the data herein in the case of aniline, the agreement between the predicted and experimental values of V_{max}^{∞} which has been discussed in (c) is evidence against an ordered ternary complex scheme in the case of quinoline. In terms of the ordered ternary complex scheme (eq 7), $V_{max}^{\infty} = k_t k_d [E]_{total}/(k_t + k_d)$. Consequently, in order to explain the agreement between the predicted and experimental values of V_{max}^{∞} for the reaction between PM-pyridinium and quinoline in terms of an ordered ternary complex scheme, it is necessary to assume the unlikely coincidence that V_{max}^{∞} for the reaction between PM-pyridinium and *p*-nitrobenzenethiol equal $k_t[E]_{total}$ for the reaction of PM-pyridinium with quinoline.

It should be noted that there are two possible types of structures for a PM-enzyme intermediate. One is a structure in which there is a covalent bond between the methylene carbon and a nucleophilic group (X) of the enzyme (I). In th caseis the enzyme functions as a nucleophilic catalyst. There is a



I



II

nonenzymatic model for such catalysis: bisulfite catalyzes the transfer of the PM group from one nitrogen heterocycle to another (Matsukawa and Yurugi, 1951a,b, 1952). It is not known whether the intermediate in this model is the sulfonate (PM-SO_3^-) or some other compound. The other structure for the enzymic intermediate is the *o*-quinone methide imine (II), which is formed by the elimination of the nitrogenous base. The hydrolysis of PM-Cl to PM-OH in water is a nonenzymatic model for this reaction pathway. Kinetic studies have shown that the hydrolysis occurs *via* rate-determining unimolecular elimination of chloride ion followed by the extremely rapid reaction of II with water (M. Mann and G. E. Lienhard, unpublished data).

Acknowledgment

I thank Misses Tung-Chia Wang and Ann Price for technical assistance.

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Isozymes of Rat Liver Mitochondrial Malate Dehydrogenase. Evidence for the Existence of Nonidentical Subunits*

K. G. Mann† and C. S. Vestling

ABSTRACT: The isozymes of rat liver mitochondrial malate dehydrogenase have been partially resolved by carboxymethyl-Sephadex chromatography. The isolated isozymes have been studied by the techniques of reversible acid dissociation and two-dimensional tryptic fingerprinting. In addition, the purified isozyme mixture has been studied by acrylamide gel electrophoresis under dissociating conditions. The results obtained from these studies indicate that rat liver mitochondrial malate dehydrogenase is composed of two nonidentical subunits.

Rat liver mitochondrial malate dehydrogenase (hereafter referred to as mitochondrial enzyme) has a native molecular weight of 66,300, and is composed of two subunits of similar or identical molecular weight (Mann and Vestling, 1969). Either crude extracts or highly purified samples of the rat liver enzyme can be resolved into five catalytically active forms by the technique of starch gel electrophoresis (Mann and Vestling, 1968). Similar observations of multiple catalytic forms of the mitochondrial enzyme have been reported for a number of species (Grimm and Doherty, 1961; Thorne *et al.*, 1963). The isozymes of mitochondrial enzyme have been given the letter designations A, B, C, D, E, beginning with the most cationic isoenzyme (Kitto *et al.*, 1966a). In the rat liver mitochondrial isozyme series A, B, and C account for about 90% of the total enzymatic activity.

Kitto *et al.* (1966b) have reported studies conducted on the separated isozymes of chicken heart mitochondrial enzyme which suggest that the mitochondrial isozymes are conformational isomers of the same polypeptide chain(s). However, Schecter and Epstein (1968) have reported reversible denaturation studies conducted on the partially resolved chicken heart isozymes which are inconsistent with the "conformer" hypothesis of Kitto *et al.* (1966a,b). A previous communication from our laboratory (Mann and Vestling, 1968) reported reversible acid dissociation studies conducted on partially resolved samples of the rat liver mitochondrial isozymes, A, B, and C, which suggested that isozymes A and C were homodimers of the types XX and YY, while isozyme B was a hybrid dimer of type XY.

We wish to report additional data which support our previous hybrid dimer hypothesis. These data involve acid hybridization, subunit electrophoresis, and fingerprint studies conducted on the partially resolved principal isozymes, mitochondrial enzymes A, B, and C from rat liver.

Materials and Methods

Rat liver mitochondrial enzyme was purified by the method of Sophianopoulos and Vestling (1962) from frozen livers supplied by Pentex. L-(1-Tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin was purchased from Worthington Biochemicals Inc.

CM-Sephadex was purchased from Pharmacia Fine Chemicals and purified as follows. The resin was allowed to swell in distilled water for 24 hr. The slurry was filtered and suspended in three volumes of 0.5 N HCl for 1 hr. The suspension was then filtered and washed with distilled water until free of chloride ion. The resin in the H^+ form was then suspended in 0.5 N NaOH for 1 hr, filtered, and washed with distilled water

* From the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52240. *Received March 30, 1970.* Supported by a grant from the National Institutes of Health (CA 07617), and by a National Aeronautics Space Administration traineeship and a predoctoral fellowship award from the U. S. Public Health Service (A-F1-GM-34,119) to K. G. M. Inquiries concerning this report should be directed to C. S. V.

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