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INHIBITION OF COENZYME ACTIVATION OF ASPARTATE AMINOTRANSFERASE*

Gina M. Hartmeier, Ashok K. Sawhney and Bob In-yu Yang+

Department of Chemistry, College of Arts and Sciences and School of Medicine
University of Missouri-Kansas City
Kansas City, Missouri 64110

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Forty compounds were surveyed for their effect on the activation of pig heart apoaspartate aminotransferase by pyridoxamine 5'-phosphate. Most of the nucleotides, sugar phosphates, coenzymes, phospholipid precursors and inorganic oxyanions tested were found to be inhibitory. With few exceptions, the only requirement for a substance to be inhibitory is the presence of a di- or polyanionic moiety analogous to the 5'-phosphate group of the cofactor. In spite of the lack of overall structural similarity to pyridoxamine 5'-phosphate, inorganic pyrophospate and apparently other inhibitors are characterized by dissociation constants comparable in magnitude to that previously reported for the natural cofactor. The physiological significance of the inhibition of coenzyme activation of apoaspartate aminotransferase by these common biological compounds is not known.

The apoenzyme of pig heart aspartate aminotransferase may be used to determine pyridoxal-P [§] and pyridoxamine-P with a detection limit of 50×10^{-15} moles (1). However, the endogenous B_6 vitamers in extracts of several animal matricies, e.g. plasma, liver and brain, have been found to activate the apoenzyme considerably more slowly than the authentic compounds in simple buffers (1). This phenomenon constitutes a significant source of error in the vitamin determination. Since different biological samples display similar effect on apoenzyme activation, substances commonly found in these samples are most likely to be responsible for the inhibition. In this report, we describe the inhibitory potential of forty organic and inorganic phosphates and other anionic compounds.

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+ To whom inquiries should be addressed.

§ The abbreviations used are as follows: -P, phosphoric residue; cAMP, adenosine 3':5'-monophosphate; cGMP, guanosine 3':5'-monophosphate; G6P, D-glucose 6-phosphate; Xu5P, D-xylulose 5-phosphate; F6P, D-fructose 6-phosphate; FDP, D-fructose 1,6-diphosphate; GAP, D-glyceraldehyde 3-phosphate; DHAP, dihydroxyacetone phosphate; 2,3-DPG, 2,3-diphospho-D-glycerate; 3-PG, D-3-phosphoglycerate; 2-PG, D-2-phosphoglycerate; PEP, phosphoenolpyruvate; TPP, thiamine pyrophosphate; phosphatidate, L-α-phosphatidate, dipalmitoyl; choline-P, O-phosphorylcholine; ethanolamine-P, O-phosphorylethanolamine; phosphotyrosine, O-phospho-DL-tyrosine.

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MATERIALS AND METHODS

MATERIALS

All purine and pyrimidine nucleotides, sugar phosphates, coenzymes and phospholipid precursors were obtained from Sigma Chemical Company. So were phosphotyrosine, L-aspartate, L-glutamate, α-ketoglutarate, ammonium sulfate, Tris hydrochloride, sodium pyrophosphate, Sephadex G-25-40, porcine heart malic dehydrogenase and holoaspartate aminotransferase. A second sample of fructose 6-P was purchased from Research Organics, Inc. Glycerol-P (20-28% alpha) was from Eastman Organic Chemicals. Sodium perchlorate, sulfate and phosphate and barium chloride were from Fisher Scientific Company.

METHODS

Preparation of apoaspartate aminotransferase. The holoenzyme was resolved by converting the pyridoxal-P form of the enzyme to its pyridoxamine-P form followed by incubation with concentrated phosphate buffer at low pH. The procedure was that of Scardi et al. (2) as modified by Furbish et al. (3). The last step, i.e. gel filtration, was performed using a column longer (1.0 x 25 cm) than previously employed in order to ensure complete removal of ammonium sulfate.

Determination of enzyme reconstituted activity. Reformation of catalytically active aspartate aminotransferase was done by mixing 0.1 mM apoenzyme in 25 mM Tris buffer, pH 8.3, with 50-fold molar excess of an aqueous solution of pyridoxal -P approximately 10 mM in concentration. Following a 30-minute incubation period at room temperature, the mixture was diluted in two steps with 25 mM Tris buffer, pH 8.3, to yield a solution that was 0.1 μM in enzyme. Fifty μl of this solution were then assayed to obtain enzyme reconstituted activity.

Determination of enzyme residual activity. Residual aminotransferase activity in the apoenzyme was calculated from the transamination rate measurement using 50 μl of 0.1 μM apoaspartate aminotransferase in the assay.

Enzyme assay. Aspartate aminotransferase activity was measured, after coupling to malic dehydrogenase, by spectrophotometrically monitoring the disappearance of NADH at 340 nm. The composition of the assay mixture was essentially that of Okamoto and Morino (4). To a semi-micro cuvette was transfered 1.0 ml of a mixture containing 10 μmol of L-aspartate, 0.1 μmol of NADH, 11 units of malic dehydrogenase and 100 μmol of Tris, pH 8.3. Ten μl of 0.3 M α-ketoglutarate were then added. The reaction was initiated by the introduction of 50 μl of 0.1 μM aspartate aminotransferase. The rate of the reaction was followed in a Varian Cary 14 uv-visible absorption spectrophotometer with its full-scale deflection set at 0.1 absorbance unit and with chart speed fixed at 0.25 to 2 in/min. All assays were performed at room temperature, i.e., 22 ± 2°. Enzyme specific activity was expressed as μmoles of products formed per min per mg of enzyme (μmol/min/mg) using 46,300 as the latter's molecular weight (5).

Measurement of inhibtion. Apoaspartate aminotransferase at 0.1 μM was incubated with 0.005 μM pyridoxamine-P in the presence and absence of a test compound at 2.5 μM at room temperature for 30 min. The mixture was buffered with 25 mM Tris, pH 8.3. Fifty μl of the mixture were added to 1010 μl of the substrate cocktail and assayed for the aminotransferase activity. The ratio of the two activities is reported.

Establishment of the time course of inhibition. In order to permit calculations of the enzyme • PP_i dissociation constant, the volume of the substrate cocktail was decreased while that of the enzyme-pyridoxamine-P-PP_i mixture was increased. Thus 5.50 nM apoenzyme was incubated at room temperature in 25 mM Tris buffer, pH 8.3, with 0.278 nM pyridoxamine-P in the presence and absence of 139 nM PP_i. At various intervals, 900 μl-aliquots of the mixture were introduced into a cuvette containg 100 μl of the substrate cocktail the composition of which was as follows: 100 mM L-aspartate, 3 mM α-ketoglutarate, 1 mM NADH, 110 units/ml malic dehydrogenase and 1M Tris

buffer, pH 8.3. The final concentrations of the reagents were comparable to those described in the preceding section. The rate of the aminotransferase reaction was measured spectrophotometrically.

Preparation of stock solutions of the test compounds. Stock solutions, 10 to 50 mM in concentration, were prepared by dissolving appropriate amounts of each substance in water. Glyceraldehyde 3-phosphate and dihydroxyacetone phosphate were obtained from their respective diethylacetal and dimethylketal derivatives by acid hydrolysis following the manufacturer's instructions. Quantitative conversion was assumed. Depending on their known stability, the compounds were tested for their inhibitory potency one to five days after their preparation. All dilutions were carried out immediately before testing.

Determination of concentration. Spectrophotometric methods were used to determine the concentration of the following substances: apoaspartate aminotransferase (3), pyridoxal-P, pyridoxamine-P (6), ATP, GTP, CTP, UTP, ADP, GDP, AMP, GMP, CMP, UMP, dTMP (7), cAMP, cGMP (8), thiamine pyrophosphate (9), riboflavin, FMN and FAD (10). Concentrations of all other compounds were calculated from their mass and solution volume.

Treatment of data. Unless otherswise noted, all values reported are the mean ±SD of three determinations.

RESULTS AND DISCUSSION

Characteristics of the apoaspartate aminotransferase. The apoenzyme prepared from the commercial holoenzyme had a concentration of 62.2 μM. It displayed a residual activity of 2.2 ± 0.3 μmol/min/mg. Upon incubation with excess pyridoxal-P, the reconstituted activity was measured to be 71 ± 1 μmol/min/mg. The latter activity was lower than obtainable from apoenzyme prepared from purer holoaspartate aminotransferase (1). This apoenzyme preparation was otherwise indistinguishable from those previously employed.

Inhibition of coenzyme activation of the apoenzyme. Forty compounds were tested for their inhibitory potential. The extents of apoenzyme activation by pyridoxamine-P in the presence and absence of a test compound are compared. The results are shown in Table 1. Five major classes of substances were examined. Of the nucleotides, all tri- di- and mononucleotides containing two or more negative charges caused inhibition. Whether the nitrogen base was a purine or pyrimidine did not appear to be a determinant. Only one deoxyribonucleotide, i.e. dTMP, was tested. However, it seemed inconsequential whether the sugar moiety was in the oxy- or deoxy- form. Phosphodiesters, e.g. cAMP and cGMP, bearing only one negative charge on the phosphate group were shown not to inhibit pyridoxamine-P activation of the apoenzyme. The group of sugar phosphates tested include nearly all the intermediates along the glycolytic pathway. All were found to be inhibitory. Characteristically, these compounds contain

Table 1. Inhibitors of coenzyme activation of apoaspartate aminotransferase[a]

Compound	%Activity remaining	Compound	%Activity remaining	Compound	%Activity remaining
Nucleotides		**Sugar phosphates**		**Coenzymes**	
ATP	35.2±1.0	G6P	76.1±1.4	CoA	32.3±1.1
GTP	-[b]	Xu5P	45.5±2.5	TPP	96.8±3.6
CTP	-[b]	F6P[c]	48.6±1.0	NADH	101.6±5.7
UTP	-[b]	FDP	28.4±1.5	$NADP^+$	76.3±0.3
ADP	47.9±2.4	GAP	27.6±1.1	Riboflavin	90.2±4.7
GDP	-[b]	DHAP	52.1±1.8	FMN	31.5±0.7
AMP	45.5±2.1	2,3-DPG	39.8±3.0	FAD	58.7±6.9
GMP	34.8±2.3	3-PG	34.2±1.4		
CMP	48.7±3.2	2-PG	57.8±1.6		
UMP	28.9±0.3	PEP	28.3±1.2		
dTMP	30.9±0.2				
cAMP	105.0±6.3	**Inorg. oxyanions**		**P-lipid precursors & misc**	
cGMP	112.7±6.3	P_i	53.7±0.8	Phosphatidate	63.9±1.3
		PP_i	33.8±1.1	Choline-P	113.8±2.7
		AsO_4^{3-}	74.3±1.3	Ethanolamine-P	101.8±4.2
		ClO_4^-	97.7±1.9	Glycerophosphate	52.7±0.3
		SO_4^{2-}	101.9±1.3	Phosphotyrosine	40.6±1.9

[a]This experiment was carried out as described in Measurement of inhibition.
[b]Only one determination was run. In all cases, the compounds tested were found to be inhibitory qualitatively.
[c]The value was obtained using F6P (barium salt) from Research Organic, Inc. F6P (disodium salt) supplied by Sigma Chemical Co. showed no inhibitory effect.

at least one dianionic phosphate moiety. The inorganic oxyanions shown may be considered structural analogs of P_i. Under the experimental conditions, i.e. pH of 8.3, these oxyanions, except perchlorate, carried two or more negative charges. Perchlorate, as expected, was noninhibitory. The anomaly in this series of anions is sulfate which also was found to be noninhibitory. Due to the facts that many enzymes are supplied as suspensions in ammonium sulfate and that the salt is used for protein fractionation, the effect of sulfate at higher concentrations was investigated. The results are shown in Table 2. Ninety percent inhibition was achieved by 10 mM sulfate. Clearly activation of the apoenzyme can be significantly affected if sufficient sulfate ions are present. As for the coenzymes, most of which are nucleotides, new inhibition patterns are suggested by the results in Table 1. As expected, compounds with a monoesterified phosphate group, i.e. CoA, $NADP^+$, FMN were found to be inhibitors. It appears that the diesterified pyrophosphoryl moiety which carries two negative charges also imparts some inhibitory

Table 2. Sulfate inhibition of coenzyme activation of apoaspartate aminotransferase[a]

Sulfate concentration	Enzyme Specific Activity
mM	μmol/min/mg
0	35.3 ± 1.1
1	27.5 ± 1.2
5	8.7 ± 0.6
10	3.4 ± 0.3

[a]This experiment was carried out as described in Measurement of inhibition. The inhibitor counterion was ammonium. The coenzyme used was 15 nM pyridoxal-P.
[b]This preparation of apoenzyme had a residual activity of 1.1 ± 0.4 μmol/min/mg (two determinations).

potential. Thus CoA, $NADP^+$ and FAD showed significant effect on apoenzyme activation. Riboflavin which possesses no anionic moiety analogous to phosphate showed only minimal inhibitory effect. NADH was present in the substrate cocktail at 0.1 mM. Conceivably it could inhibit the aminotransferase to some extent. As a test compound, NADH was present at a much lower concentration. Whatever inhibition it exerted would have been completely masked, thus the apparent absence of inhibition seen in Table 1. Lack of inhibition by thiamine pyrophophate might be due to the presence of a positively charged nitrogen atom in the vicinity of the anionic moiety. This arrangement of electrical charges is seen in several other non-inhibitory compounds described below. Of the four phospholipid precursors tested, phosphatidic acid and glycerophosphate showed inhibition as expected. Choline-P and ethanolamine-P proved to be noninhibitory although both contain a dianionic phosphomonoester moiety. As in thiamine pyrophosphate, these substances are characterized by the presence of a positively charged nitrogen atom in the vicinity of the negatively charged phosphate group. Finally phosphotyrosine was found to be inhibitory. In this compound, a zwitterion rather than a simple cation is centered around the α-carbon.

Effect of cations on coenzyme activation of the apoenzyme. The majority of the anions studied were supplied as salts of the following counterions: sodium, barium and ammonium. These cations were found to be noninhibitory (Table 3). Calcium ion did not inhibit apoenzyme activation either. In fact, several of the cations shown appear to be stimulatory. Therefore, the inhibitory property observed could be attributed entirely to the anions as described.

Table 3. Effect of cations on coenzyme activation of apoaspartate aminotransferase[a]

Cation[b]	Concentration[c]	% Activity remaining
Sodium	97 mM	104 ± 3[d]
Ammonium	20 mM	144 ± 7
Barium	2.5 μM	112 ± 2
Calcium	49 mM	121 ± 9[d]

[a]Unless otherwise specified, this experiment was carried out as described in Measurement of inhibition.
[b]The counterion in each case was chloride.
[c]The values for ammonium and barium ions represent the highest concentrations encountered in this study. The concentrations of sodium and calcium ions were arbitrary.
[d]A separate apoenzyme preparation was used. It had a residual activity of 0.86 ± 0.11 μmol/min/mg and a reconstituted activity in the presence of excess pyridoxal-P of 171 ± 7 μmol/min/mg. The coenzyme used in testing the cation effect was pyridoxal-P at 15 nM.

Time course of PP_i inhibition. In order to more rigorously compare the affinity of the inhibitors and pyridoxamine-P for the apoenzyme, determination of the dissociation constant of the apoenzyme•PP_i complex was attempted. Attainment of equilibrium was a prerequisite. Furthermore, the equilibrated mixture of apoenzyme, pyridoxamine-P and PP_i had to remain essentially unperturbed as the substrate cocktail was introduced. Otherwise, the aminotransferase activity measured would not necessarily reflect the equilibrium concentration of the apoenzyme • pyridoxamine-P complex. Experimentally the reconstitution mixture was diluted only 1.1-fold when it wass assayed. Figure 1 shows the time course of pyridoxamine-P activation of the apoenzyme in the presence and absence of PP_i. Because of the very low concentrations of both the apoenzyme and pyridoxamine-P, prolonged incubation was required to achieve maximum reconstitution. The gradual decrease in enzyme specific activity beginning at the fifth hour might be due to deterioration of the substrate cocktail, apoenzyme or pyridoxamine-P. Assuming that pyridoxamine-P and PP_i bind the apoenzyme competitively and that apoenzyme • pyridoxamine-P complex is the only catalytically competent species, the following equations may be readily derived.

$$K_i = \frac{(A-X-Y)\ (C-Y)}{Y} \quad \text{.....Eq. 1}$$

where

$$Y = \frac{(A-X)\ (B-X)-K_d \cdot X}{B-X} \quad \text{.....Eq. 2}$$

X = equilibrium concentration of apoenzyme • pyridoxamine-P complex

Y = equilibrium concentration of apoenzyme• PPi complex

A = total enzyme concentration

B = total pyridoxamine-P concentration

C = total PP_i concentration

K_d = dissociation constant of apoenzyme • pyridoxamine-P complex

K_i = dissociation constant of apoenzyme • PP_i complex

Thus K_i may be calculated if the remaining six parameters are known. A, B, and C are experimentally assigned values. X is the product of the fraction of activity remaining and the equilibrium concentration of apoenzyme • pyridoxamine-P complex in the absence of PP_i. From Figure 1, the fraction of activity remaining at equilibrium may

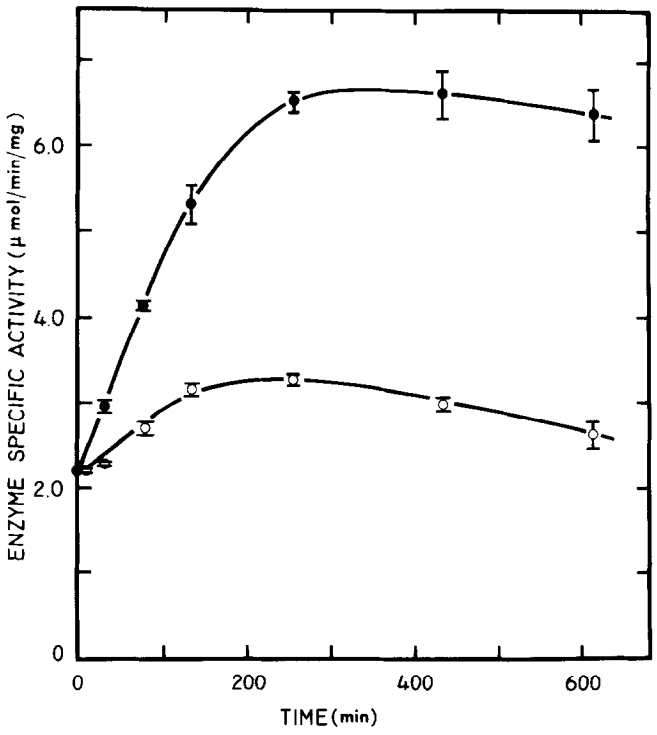

Figure 1. Time course of coenzyme activation of apoaspartate aminotransferase in the presence and absence of PP_i. The experiment was carried out as described in Establishment of the time course of inhibition. O——O, with PP_i; ●——●, without PP_i. The vertical bars represent standard deviation.

be calculated to be 0.492. The equilibrium concentration of apoenzyme • pyridoxamine-P complex in the absence of PP_i, Z, may be obtained from Eq. 3. Y may be computed from Eq. 2. The sixth parameter, i.e. K_d, has been reported to be 67 nM for pyridoxamine-P

$$K_d = \frac{(A-Z)\ (B-Z)}{Z} \quad \text{.....Eq. 3}$$

(11). Using Eq. 1, K_i is computed to be 120 nM. Thus PP_i binds the apoenzyme nearly as tightly as does the natural cofactor. Several sources of error should be noted. First, the apoenzyme was not pure, thus the total apoenzyme concentration was undoubtedly lower than assumed. Second, the apoenzyme contained an undetermined amount of pyridoxal-P or pyridoxamine-P. The residual activity was ignored in the calculation. Third, the buffer used in attaining equilibrium is different from that used in obtaining the K_d.

The inhibitory effect of several of the anions had been anticipated from their ability to effect resolution of the pyridoxamine-P form of the enzyme. Scardi et al. (2) showed that phosphate and sulfate could be used to remove pyridoxamine-P from the enzyme. It was postulated that the anions competed with the 5'-phosphate moiety of the coenzyme for a cationic site on the apoenzyme. In this report, we have shown that numerous di- and polyanions, structurally unrealted to pyridoxamine-P, inhibit coenzyme activation of the apoenzyme. It seems likely, for some of the inhibitors at least, that the inhibition is of the competitive type, with respect to the coenzyme. Also it appears that substances considerably bulkier than pyridoxamine-P can be readily accommodated at the coenzyme binding site. In this communication, we have found the inhibitors to be active in micromolar concentrations. In humans, the serum level of phosphate and sulfate is 2-5 mEq/liter. The concentration in intracellular fluids is approximately 150 mEq/liter (12). These values are far in excess of those employed. Thus the possibility exists that aspartate aminotransferase may not be functioning physiologically at its maximal capacity. Existence of the apo form of the enzyme may also be a consequence of the type of inhibition presented here.

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