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N-(PHOSPHONACETYL)-L-ASPARTATE INHIBITION OF THE ENZYME COMPLEX OF PYRIMIDINE BIOSYNTHESIS*

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Abstract—The inhibition of aspartate carbamoyltransferase (ACTase) from rat Novikoff tumor by *N*-(phosphonacetyl)-L-aspartate (PALA) was studied in a substrate mixture permitting endogenous synthesis of carbamoyl phosphate. Among the components required for carbamoyl phosphate synthetase activity, ATP, $Mg(C_2H_3O_2)_2$ and KCl interfered with inhibition by PALA (with added carbamoyl phosphate). The inhibition was also decreased when the concentration of partially purified enzyme was increased. In the system dependent on carbamoyl phosphate synthetase, the 50% inhibitory concentration of PALA was lower than that in the same mixture plus 0.2 mM carbamoyl phosphate, but higher than in the usual simple assay mixture with 0.2 mM carbamoyl phosphate.

These experiments were undertaken to explain why *N*-(phosphonacetyl)-L-aspartate (PALA)† failed to inhibit pyrimidine biosynthesis *in vivo* as strongly as would be expected from its effects *in vitro*, despite an apparently effective concentration of the drug in the tissues. As reported in the accompanying paper [1], in biopsy specimens from seven patients in which we determined both PALA and pyrimidine nucleotide concentrations, the former ranged from 3.6 to 89 μg/g of tissue, at times from 6 to 24 hr after PALA (or, in two cases, 1.5 or 4 hr after the second day's dose). This is well above the 0.26 μg/ml (1 μM) or 1.5 μg/ml (6 μM) required for 90% inhibition of a partially purified ACTase from human spleen, measured at 0.2 or 1.0 mM carbamoyl phosphate. Yet the pyrimidine nucleotides had decreased only 29–72%. In addition, in four out of seven post-treatment biopsy specimens, the measured ACTase activity was higher than expected based on the concentration of PALA contributed by the tissue to the assay; the data are plotted in Fig. 1 to illustrate this.

PALA inhibits aspartate carbamoyltransferase (ACTase) (EC 2.1.3.2), the second enzyme in the biosynthesis of pyrimidine nucleotides, competitively with the substrate carbamoyl phosphate; that is, at higher concentrations of carbamoyl phosphate, PALA is less effective. Carbamoyl phosphate is produced by the first enzyme of the pathway, carbamoyl phosphate synthetase (EC 2.7.2.9). In mammals, the first three enzymes of this pathway occur as activities of an enzyme complex, sometimes a single protein chain [2, 3], so that carbamoyl phosphate is produced and utilized within a very small volume. Therefore, it is difficult to predict or measure directly its effective concentration at the enzyme active site. We decided to measure inhibition by PALA *in vitro* under conditions such that ACTase was dependent on endogenously synthesized carbamoyl phosphate. Since the carbamoyl phosphate synthetase activity, measured *in vitro*, is much lower (and less stable) than the ACTase activity of the same enzyme preparation [2], a much higher level of enzyme protein

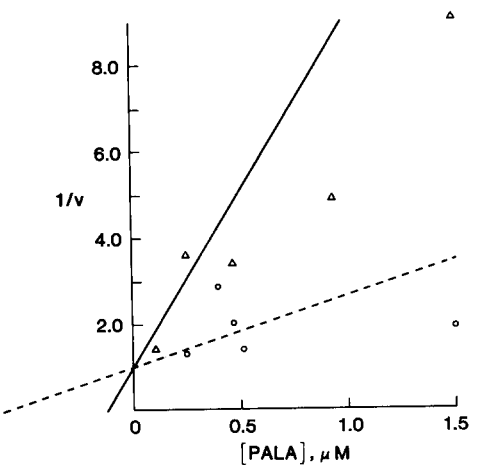

Fig. 1. Apparent inhibition of ACTase in extracts of biopsy specimens [1]. Ordinate: reciprocal of relative activity; that is, ACTase activity of extract from pre-treatment specimen divided by apparent ACTase activity of extract from post-treatment specimen. Abscissa: PALA concentration in assay mixture contributed by the extract of the post-treatment specimen. The triangles represent activity assayed with 0.2 mM carbamoyl phosphate; the circles represent activity assayed with 1.0 mM carbamoyl phosphate. The lines are standard inhibition curves [1] obtained with partially purified ACTase from human spleen; dashed line at 1.0 mM and solid line at 0.2 mM carbamoyl phosphate.

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† Abbreviations: PALA, *N*-(phosphonacetyl)-L-aspartate (NSC 224131); ACTase, aspartate carbamoyltransferase (EC 2.1.3.2); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; and IC_{50}, concentration of PALA giving 50% inhibition of ACTase activity.

was required. The substrate mixture was also more complex. Both of these factors were found to affect the results.

METHODS

Enzyme complex was prepared from Novikoff ascites tumor cells grown in rats. The procedure of Mori and Tatibana [2] was followed through the second ammonium sulfate precipitation; ammonium sulfate was removed on a small column of Sephadex G75 equilibrated with "Buffer B" [2], containing 30% dimethyl sulfoxide, 5% glycerol, 2 mM potassium phosphate, 1 mM dithiothreitol, and 0.5 mM EDTA, adjusted to pH 7.0. The ACTase activity was purified about 6-fold. The enzyme was stored at −80°. All the results presented here were obtained with a single enzyme preparation, which was desalted in three portions. The enzyme was diluted in Buffer B.

ACTase was assayed by the procedure we used earlier [4], with [^{14}C]aspartate as the substrate. The incubation mixture for endogenous carbamoyl phosphate synthesis, based on that of Tatibana and Shigesada [5], contained in addition to 1/5 volume of enzyme in Buffer B, 5 mM ATP, 8 mM $Mg(C_2H_3O_2)_2$, 20 mM KCl, 3.3 mM glutamine, 0.05 mM phosphoribosyl pyrophosphate, 16.7 mM $NaHCO_3$, 50 mM potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 1% glycerol, 1 mM dithiothreitol and 1.3 mM [^{14}C]aspartate (850 dpm/nmole), in a total volume of 150 μl or 300 μl. The substrate mixture was adjusted to pH 7.1 A blank lacking one substrate and another without enzyme were included in each experiment. Care was taken to warm the incubation mixture before adding the enzyme [5].

The 50% inhibitory concentration of PALA was calculated according to a Dixon plot [6]. The inhibited activity was expressed as a fraction of the control activity. The linear regression of the reciprocal of the relative activity versus PALA concentration was calculated and the 50% inhibitory concentration (IC_{50}) was determined from the equation. Samples showing more than 80% inhibition were omitted from the calculation.

RESULTS

An initial trial with the higher ATP, $Mg(C_2H_3O_2)_2$, and KCl concentrations recommended by Tatibana and Shigesada revealed that the activity in the complex synthetase-dependent system was far lower but far less sensitive to PALA than that in the simple HEPES–carbamoyl phosphate (0.2 mM)–aspartate mixture. Various components of the mixture were then tested separately, with added carbamoyl phosphate. Glycerol, dimethyl sulfoxide, phosphate, and EDTA, the components contributed by the Buffer B in the enzyme solution, increased the ACTase activity about 30% but did not affect the inhibtion by PALA when enzyme diluted 1:30 in HEPES was compared in the simple reaction mixture with enzyme diluted in Buffer B. Omission of dithiothreitol, glutamine and phosphoribosyl pyrophosphate from the complex mixture (with carbamoyl phosphate and dilute enzyme) did not change the PALA inhibition curve. ATP, KCl and especially $Mg(C_2H_3O_2)_2$ interfered with the PALA inhibition when added to the simple mixture (Table 1). KCl also decreased the activity of ACTase. Reducing the concentration of these components from the recommended [5] levels to those we used decreased the interference but also decreased the synthetase activity somewhat. In a separate experiment, $MgCl_2$ interfered with inhibition by PALA slightly more than did $Mg(C_2H_3O_2)_2$, while $KC_2H_3O_2$ had almost the same effect on the inhibition as KCl. Therefore, the interference by $Mg(C_2H_3O_2)_2$ was due to the magnesium and not to acetate.

With the revised incubation mixture described above, inhibition by PALA in the presence of different concentrations of carbamoyl phosphate was compared to that in the synthetase-dependent system. When carbamoyl phosphate was added, either glutamine or bicarbonate was omitted. Four to six concentrations of PALA were tested in each condition. The results are summarized in Table 2.

The inhibition by PALA, expressed as the IC_{50}, was affected not only by the carbamoyl phosphate concentration but also by the enzyme amount and by the complex reaction mixture. The uninhibited activity did not vary greatly with carbamoyl phosphate concentration in the range from 0.2 to 1.0 mM;

Table 1. Interference with inhibition by PALA*

Addition	Activity (nmoles) Control	+PALA	% of Control
None	1.41	0.36	25
KCl, 20 mM	1.15	0.39	34
KCl, 50 mM	0.91	0.36	40
ATP, 5 mM	1.37	0.42	31
ATP, 10 mM	1.39	0.45	32
$Mg(C_2H_3O_2)_2$, 8 mM	1.44	1.05	73
$Mg(C_2H_3O_2)_2$, 15 mM	1.43	1.14	80
All 3, low level	1.10	0.72	66
All 3, high level	0.71	0.58	81

* All assays contained HEPES, 1.25 mM [^{14}C]aspartate, 0.2 mM carbamoyl phosphate and enzyme solution which contributed dimethyl sulfoxide, glycerol, EDTA, and dithiothreitol. The PALA concentration, when present, was 0.08 μM. Each entry is the average of duplicate samples.

Table 2. Variation of 50% inhibitory concentration of PALA with experimental conditions

Mix*	Enzyme (μg)	Carbamoyl phosphate (mM)	Uninhibited activity† (pmoles·min^{-1}·μg^{-1})		PALA IC_{50}‡ (μM)
Simple	13–26	0.2	12.7 (12.1–13.2)	(2)	0.02
		1.0	11.3	(1)	0.14
Complex	13–18	0.1	10.8	(1)	0.04
		0.2	11.8 (10.7–14.2)	(4)	0.11
		0.5	12.7 (11.3–15.4)	(3)	0.29
		1.0	11.1	(1)	0.62
Complex	88–226	0.2	10.9	(1)	0.36
		0.5	13.2 (11.7–14.7)	(2)	0.62
		1.0	12.3	(1)	1.38
Complete	130–264	0	0.55 (0.39–0.81)	(5)	0.058 (0.035–0.10)

* The simple mix contained only HEPES, aspartate, carbamoyl phosphate and enzyme in Buffer B. The complete mix contained the substrates, activators and protectants listed in the text. The complex mix was the same as the complete except that bicarbonate was omitted in one experiment and glutamine was omitted in the remaining three experiments.

† The average activity, range and number of experiments are shown.

‡ The 50% inhibitory concentration (IC_{50}) was calculated (by the least squares method) from the pooled data (concentration versus the reciprocal of inhibited activity expressed as a fraction of the uninhibited activity) from the number of experiments shown, except in the case of the complete mix. Those experiments were calculated separately and the results were averaged. For the other conditions, the IC_{50} values calculated separately differed by less than 80% at 0.5 mM carbamoyl phosphate and less than 20% at 0.2 mM.

this is well above the K_m (47 μM). As expected, the IC_{50} increased with the carbamoyl phosphate concentration in both reaction mixtures. Likewise, the ACTase activity per min per mg protein, with 0.2 to 1.0 mM carbamoyl phosphate, changed less than 10% over a 5- to 15-fold range of protein concentration, but the IC_{50} increased 2- to 3-fold. The IC_{50} with 0.2 mM carbamoyl phosphate (with a constant enzyme amount) was quite consistent between experiments, both in the complex mixture and in the simple mixture. The IC_{50} was four to six times higher in the complex mix than in the simple mix at the same carbamoyl phosphate concentration.

The IC_{50} for PALA in the synthetase-dependent system was lower in all five experiments than that with 0.2 mM carbamoyl phosphate in the complex mix at high enzyme concentration. In general, it was similar to that for the complex system with 0.1 or 0.2 mM carbamoyl phosphate at low enzyme concentration.

DISCUSSION

To relate these results to the situation *in vivo* is not simple. The concentration of ATP in Novikoff cells is probably less than that in our mixture*, but numerous other compounds are present also. The free magnesium concentration in the cell is probably lower. The concentration of the enzyme complex is probably higher *in vivo*; a rough calculation from the activity in cell extracts indicates that its concentration in Novikoff cells may be five or ten times the highest that was used.

* R. B. Hurlbert and E. C. Moore, unpublished observations.

The data in Table 2 would suggest that the effective endogenous carbamoyl phosphate concentration in our reaction mixture was below 0.1 mM. However, this would presumably be higher with the higher enzyme concentration *in vivo*. Loss of the unstable carbamoyl phosphate synthetase activity during purification would also lead to underestimating the effective concentration of its product. On the other hand, carbamoyl phosphate synthetase is believed to be feedback regulated *in vivo* by pyrimidine nucleotides [2], which would tend to decrease the concentration of its product as long as the nucleotide level remained high. It is also likely that carbamoyl phosphate can accumulate locally if ACTase is inhibited, and tend to reverse the inhibition. An increased activity of carbamoyl phosphate synthetase has been associated with resistance to PALA [7].

The observed effect of enzyme concentration on inhibition by PALA even in the presence of exogenous carbamoyl phosphate may partially explain the resistance to PALA of tumors having high ACTase activity.

The combined effects of salts and other compounds, enzyme concentration and possible carbamoyl phosphate accumulation would all tend to decrease the sensitivity of ACTase to PALA. It seems likely, therefore, that its inhibition *in vivo* requires higher PALA concentrations than would be predicted from the results *in vitro* with 0.2 mM carbamoyl phosphate in a simple incubation mixture. How much higher remains uncertain. Of course, I do not intend to suggest that this is the only mechanism of resistance to PALA; other factors such as increased synthesis of ACTase and the salvage of uracil, uridine and cytidine are probably of equal or greater importance.

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