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BINDING OF RADIOLABELED *N*-(PHOSPHONACETYL)-L-ASPARTATE TO ASPARTATE TRANSCARBAMYLASE FROM EHRLICH ASCITES TUMOR CELLS*

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Abstract—Binding of *N*-(phosphonacetyl)-[^{3}H]L-aspartate (PALA) to aspartate transcarbamylase (ATCase, EC 2.1.3.1) in crude extracts from Ehrlich ascites tumor cells was examined. At pH 7.4, the dissociation constant was 1.39 ± 0.22 nM; the maximal binding capacity indicated an average intracellular ATCase concentration of 0.13 μM. The presence of phosphate, $MgCl_2$, or $CaCl_2$ increased the apparent dissociation constant for [^{3}H]PALA without altering the maximal binding capacity. Phosphate, a product of the ATCase reaction, probably acts as a competitor for the PALA binding site; Mg^{2+} and Ca^{2+} may inhibit [^{3}H]PALA binding by forming a chelate which reduces the effective concentration of the free [^{3}H]PALA. Carbamyl phosphate was a relatively weak inhibitor of [^{3}H]PALA binding in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer alone. Addition of NaF, an inhibitor of nonspecific phosphatases, decreased the carbamyl phosphate "K_i" as an inhibitor of [^{3}H]PALA binding to 7 μM, a value close to the K_m. NaF appears to act as an inhibitor of carbamyl phosphatase activity present in the cell extract. A first-order dissociation rate constant of 0.050 ± 0.004 min^{-1} ($T_{\frac{1}{2}}$ = 14 min) was determined by following displacement of [^{3}H]PALA with excess unlabeled PALA. The dissociation rate was strongly temperature dependent. A second-order rate constant of 3.6×10^7 liters mol^{-1} min^{-1} was calculated from this rate constant and the dissociation constant. Using these kinetic constants, a simple computer model predicted that PALA binding to ATCase is 95% complete within 14 min under the conditions of the assay; at intracellular ATCase concentrations, binding is slightly faster. These results are discussed in the context of both the kinetics of inhibition and the reversal of inhibition of pyrimidine synthesis within the intact cell.

N-(Phosphonacetyl)-L-aspartate (PALA)‡ is a rationally designed anticancer agent whose structure resembles the transition state intermediate for aspartate transcarbamylase (ATCase). PALA is a potent inhibitor of this enzyme and thus of *de novo* pyrimidine synthesis in whole cells. Numerous reports have shown inhibition to be competitive with carbamyl phosphate, the substrate which binds first in the ordered reaction sequence [1]. K_m values for carbamyl phosphate have varied from low micromolar (3–10 μM) [1–5] to about 1 mM [6]. Estimates of the K_i for PALA have ranged from low nanomolar (0.4–5 nM [2, 4, 5]) to intermediate (11 nM [7], 26 nM [1]) to relatively high values (100 nM [6]). Although these enzymes were prepared from disparate mouse, hamster, and human cell types, the variation is unusual. This variation is of interest since PALA often seems to be less potent as an inhibitor of cellular pyrimidine synthesis than might be expected [7, 8]. For example, incubation of Ehrlich ascites cells for 90 min with 200 μM PALA generated sufficient intracellular PALA to give a concentration of 2.7 μM, assuming even distribution in the cytoplasm, but only 70% inhibition of *de novo* pyrimidine synthesis [8]. This result is partially due to excess ATCase activity as compared to that of carbamyl phosphate synthetase, the rate-controlling enzyme for the pathway. To make ATCase activity rate-limiting, a large fraction of this enzyme must be inhibited. Less clear is the role of competition by carbamyl phosphate which may accumulate behind the block and overcome the effects of low PALA concentrations. Accumulation of carbamyl phosphate has been shown to occur when these enzymes are incubated *in vitro* [3], but this has not been demonstrated within intact cells. Carbamyl phosphate is unstable in aqueous solutions [3] and may be subject to hydrolysis by intracellular phosphatases [3, 9]. Once inhibition of pyrimidine synthesis is achieved, the effects of PALA are long-lasting. Tsuboi *et al.* [10] showed that, after exposure of cells in culture to 1 mM PALA for 24 hr, *de novo* pyrimidine synthesis was undetectable for at least 6 days. Following cessation of PALA therapy, ATCase activity was less than 50% of pretreatment levels in human peripheral leukocytes [4] or mouse liver [11] for more than 3 weeks.

The investigations reported here explore the binding of [^{3}H]PALA to ATCase present in crude extracts of Ehrlich ascites tumor cells. These results suggest that some discrepancies in the kinetics of ATCase might be related to effects of some common buffer salts on PALA binding and to the activity of

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‡ Abbreviations: PALA, *N*-(phosphonacetyl)-L-aspartate; ATCase, aspartate transcarbamylase, EC 2.1.3.2; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

carbamyl phosphatase in cell extracts that rapidly lowers the actual concentration of carbamyl phosphate. Evidence is also presented which demonstrates that, on the time scale of inhibition of pyrimidine synthesis observed in cell culture [10] and *in vivo* [4, 11], binding and release of PALA from ATCase are rapid. The cellular kinetics of inhibition are more readily accounted for on the basis of PALA's recently described [8] unique mechanism of uptake by endocytosis and pH-dependent escape from lysosomes which results in irreversible trapping in the cytoplasm.

MATERIALS AND METHODS

Chemicals. [^{3}H]PALA was synthesized from [^{3}H] aspartate (8 Ci/mmole) (Amersham Corp., Arlington Heights, IL) as described previously [8]. Solutions of dilithium carbamyl phosphate (Sigma Chemical Co., St. Louis, MO) were prepared just before use and kept at 0°.

ATCase. Ehrlich ascites cells were maintained in CF1 mice as described [8]. Cells were washed in saline and suspended at a density of 10^8/ml in 0.05 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, containing 0.1% Triton X-100. The suspension was frozen and thawed three times, mixed vigorously on a vortex, and then centrifuged at 2000 *g* for 10 min to remove cell debris. Inclusion of Triton X-100 improved the recovery and reproducibility of the freeze–thaw extraction procedure but had no effect on [^{3}H]PALA binding or ATCase activity.

Methods. [^{3}H]PALA binding was measured by the column centrifugation method of Penefsky [12] as modified by Fry *et al.* [13]. [^{3}H]PALA and other solutes were prepared in 0.05 M HEPES, pH 7.4. ATCase extract (30–75 μl) was mixed with [^{3}H] PALA and buffer to give a final volume of 150 μl. The samples were incubated at 37° for 30 min to achieve equilibrium binding. A column of Sephadex G50 (Sigma) was prepared in a tuberculin syringe and centrifuged for 5 min at 1000 *g*. To separate bound [^{3}H]PALA from free [^{3}H]PALA, 100 μl of the mixture was applied to a column and immediately centrifuged at 0° for 5 min at 50–100 *g*, and then for 5 min at 1000 *g*. The effluent containing bound [^{3}H] PALA was collected directly into a 4-ml scintillation vial. A 30-μl aliquot of the [^{3}H]PALA–ATCase mixture was counted directly. The concentration of free PALA was determined by subtracting the bound concentration from the total. In experiments where [^{3}H]PALA was varied, binding constants were determined by nonlinear regression using a computer program in BASIC written by Duggleby [14] to fit the data directly to this equation:

$$[\text{Bound PALA}] = \frac{[\text{Maximum binding capacity}] \times [\text{Free PALA}]}{[\text{Free PALA}] + \text{Dissociation constant}}$$

Similarly, data on the dissociation of [^{3}H]PALA from ATCase was directly fit to the equation for first-order decay by a modification of the same program.

$$[\text{Bound PALA}] = [\text{Bound PALA at } t = 0] \times e^{-kt}$$

ATCase activity was assayed by the method of Porter *et al.* [15] as modified by Friedman *et al.* [16]. Computer simulations of PALA binding used the principles of network thermodynamics and the circuit simulation program SPICE2 as described in detail previously for methotrexate binding to dihydrofolate reductase [17, 18].

RESULTS

Figure 1 shows the dependence of [^{3}H]PALA binding on the concentration of free [^{3}H]PALA and the effects of some ions commonly found in assay buffers. In HEPES buffer, pH 7.4, the average dissociation constant for [^{3}H]PALA binding was 1.39 ± 0.22 nM (S.E., N = 10). This value is similar to the lowest values reported for the K_i of PALA as an inhibitor of ATCase but is considerably less than some estimates. The mean binding capacity was 0.14 pmole/10^6 cells which corresponds to a mean intracellular concentration of 0.13 μM (assuming 1.07 μl/10^6 cells [19]). We have confirmed the observation of Moore *et al.* [20] that Mg^{2+} and phosphate increase the concentration of PALA required for 50% inhibition of ATCase activity. The data in Fig. 1 indicate that phosphate, Mg^{2+}, and Ca^{2+} decreased the apparent affinity of PALA for ATCase with K_i values as competitive inhibitors of 6.6, 2.8 and 5.9 mM respectively. Higher concentrations of Mg^{2+} or Ca^{2+} caused precipitation of protein in our cell extracts and decreased maximal [^{3}H]PALA binding. From these inhibition constants, it may be calculated that the apparent dissociation constant of PALA binding to ATCase in a buffer consisting of 50 mM potassium phosphate and 10 mM $MgCl_2$ would be 17 nM, a value that is 12-fold higher than the dissociation constant in HEPES alone.

Figure 2 is a representative experiment showing

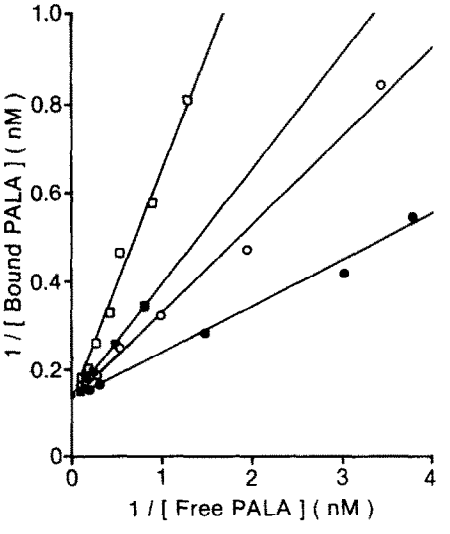

Fig. 1. Inhibition of [^{3}H]PALA binding to ATCase by ions. Binding of [^{3}H]PALA to ATCase present in crude extracts from Ehrlich ascites tumor cells was measured in 50 mM HEPES buffer, pH 7.4 (●), or in buffer plus 5 mM $CaCl_2$ (○), 5 mM $MgCl_2$ (■), or 30 mM K_2HPO_4/KH_2PO_4, pH 7.4 (□).

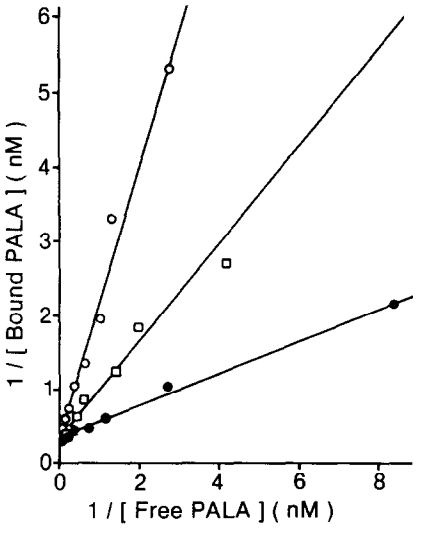

Fig. 2. Inhibition of [^{3}H]PALA binding by carbamyl phosphate. Binding of [^{3}H]PALA was measured in HEPES buffer alone (●), buffer plus 40 μM carbamyl phosphate (□), or buffer plus 40 μM carbamyl phosphate and 200 mM NaF (○).

the effects of carbamyl phosphate on [^{3}H]PALA binding. When cell extracts were incubated at 37° for 30 min with 40 μM carbamyl phosphate and various concentrations of [^{3}H]PALA, the apparent "K_i" of carbamyl phosphate was 26 μM. Addition of 200 mM NaF decreased the apparent "K_i" to 7 μM, indicating more potent inhibition of [^{3}H]PALA binding by carbamyl phosphate. NaF alone had no effect on [^{3}H]PALA binding. NaF is frequently included in a variety of assays, e.g. for kinases, to inhibit nonspecific phosphatases. To confirm the hypothesis that NaF could protect carbamyl phosphate, limiting carbamyl phosphate was incubated with excess [^{14}C]-aspartate until product formation was complete (30–40 min). NaF (150 mM) significantly increased [^{14}C]-carbamyl-aspartate formation from 34 to about 50% of the initial carbamyl phosphate. The remaining 50% is probably accounted for by carbamyl phosphatase activity that is insensitive to NaF, nonenzymatic breakdown ($T_{\frac{1}{2}}$ = 38 min [3]), and impurities in the carbamyl phosphate powder. Thus, protection of carbamyl phosphate in Fig. 2 by NaF is incomplete; the true "K_i" of carbamyl phosphate as an inhibitor of [^{3}H]PALA binding is probably somewhat lower than the value given above.

Figure 3 shows the temperature dependence of the dissociation rate of [^{3}H]PALA from ATCase. The ATCase preparations containing 5 nM [^{3}H]PALA binding sites were incubated at 37° for 30 min with 100 nM [^{3}H]PALA. At intervals, unlabeled PALA was added to a final concentration of 100 μM to prevent reassociation of [^{3}H]PALA after it dissociated from the complex. Bound [^{3}H]PALA was separated from all the samples at once. The plot shows the amount of bound [^{3}H]PALA as a function of the time of exposure to unlabeled PALA. The rate of dissociation of [^{3}H]PALA was temperature dependent. The first-order rate constants were 0.050 ± 0.004 min^{-1} (S.E., N = 3) at 37°, 0.0099 min^{-1} at 24°, and 0.0028 min^{-1} at 0°; corresponding $T_{\frac{1}{2}}$ values were 14, 70 and 245 min respectively. A second-order association rate constant of 3.6 × 10^7 liters M^{-1} min^{-1} may be calculated from the dissociation rate constant and the equilibrium dissociation constant at 37°. When these values are used to prepare a simple computer model [17, 18], simulation of the admixture of 5 nM PALA and 5 nM ATCase indicates that PALA binding is within 95% of the predicted steady state within 13 min. This is in agreement with our experimental observations (not shown). Steady-state binding is achieved somewhat more slowly at lower PALA concentrations; thus, our 30-min incubation at 37° was appropriate for measuring equilibrium binding.

DISCUSSION

Direct binding of [^{3}H]PALA was examined in order to approach the mechanism of inhibition by PALA of ATCase and cellular pyrimidine synthesis from a different perspective. Titration of ATCase binding sites with high specific activity [^{3}H]PALA has been reported by Kempe *et al.* [2] and Coleman *et al.* [21], but the kinetics of binding and factors which influence equilibrium binding have not been examined.

Some ions that are common in biological buffers were shown to increase the apparent dissociation constant for PALA binding. Phosphate, a product of the reaction, has been shown previously to compete with carbamyl phosphate to inhibit ATCase [1]. Thus, it was not surprising that phosphate was a competitive inhibitor of PALA binding. Moore [20] observed that Mg^{2+} increases the concentration of PALA required for 50% inhibition of ATCase. Our results appear to indicate competitive inhibition of PALA binding by both Mg^{2+} and Ca^{2+}. However, an alternative explanation may be that these ions form an ionic complex with PALA, thus reducing the effective concentration of free PALA. This view is consistent with the observations by Ardalan *et*

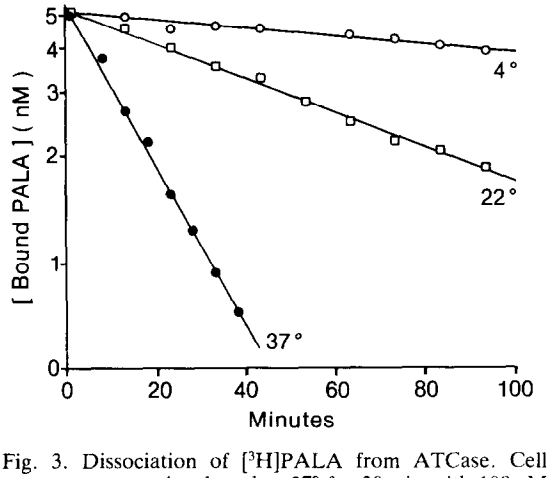

Fig. 3. Dissociation of [^{3}H]PALA from ATCase. Cell extracts were preincubated at 37° for 30 min with 100 nM [^{3}H]PALA. At intervals, unlabeled PALA (100 μM) was added, and samples were incubated at 37° (●), 24° (□), or 0° (○). Bound [^{3}H]PALA was separated from free [^{3}H] PALA for all samples at the same time.

al. [22] that PALA binds tightly to bone and is a solubilizer of bone at high concentrations.

Since inhibition of ATCase by PALA is competitive with carbamyl phosphate, we anticipated that this substrate would act competitively on [^{3}H] PALA binding; however, our initial estimates of the carbamyl phosphate "K_i" were higher than most esimates of the carbamyl phosphate K_m. Christopherson and Jones [3] showed that carbamyl phosphate spontaneously breaks down in aqueous solution at pH 7.4 and 37° with a $T_{\frac{1}{2}}$ of 37.6 min. Thus, during our 30-min incubation period with carbamyl phosphate and [^{3}H]PALA, approximately half of the original carbamyl phosphate would disappear nonenzymatically. These authors also demonstrated the existence of a carbamyl phosphatase in crude extracts of Ehrlich ascites cells [3]. Black and Jones [9] later showed that this activity, though most concentrated in the lysosomes, was distributed throughout the cell. We observed that addition of NaF, which is commonly employed in assays of kinases, etc., to inhibit nonspecific phosphatases, increased the potency of carbamyl phosphate as an inhibitor of PALA binding. Our results confirm the existence of a carbamyl phosphatase. This enzyme activity may prevent the accumulation of high levels of carbamyl phosphate behind the metabolic block which, theoretically, could overcome the competitive inhibition by PALA. The variation in reports of the carbamyl phosphate K_m may be due to failure to recognize the rapid enzymatic and non-enzymatic breakdown of carbamyl phosphate.

The dissociation of PALA from ATCase was found to be temperature dependent. The rate of release of PALA from ATCase at 22° was 5-fold slower than at 37° ($T_{\frac{1}{2}}$ of 14 min). From the first-order rate constant and the dissociation constant, we were able to calculate a second-order association rate constant and simulate by a computer model [17, 18] the approach to equilibrium at a variety of initial PALA and free ATCase concentrations. Near steady-state binding (95%) was always achieved within 15 min. Thus, the rate of PALA binding and dissociation is quite rapid compared to the time course of inhibition and recovery of pyrimidine synthesis in the intact cells. The kinetics of the interaction of PALA with cells are probably determined instead by the rates of uptake and efflux. We have recently presented evidence for uptake of PALA by a process of fluid-phase endocytosis which results in the initial sequestration of PALA in endosomes and, ultimately, in lysosomes [8]. We noted that the concentration of PALA acting on ATCase in the cytoplasm could not be inferred from measurement of total cellular PALA since a large fraction of the drug may remain latent in vesicles for long periods. At the acidic pH of the lysosome, partial protonation of the charged groups on PALA results in the reduced charge density and increase lipid solubility that are necessary for diffusion across the lysosomal membrane. Once PALA is released into the cytoplasm and re-ionized at cytoplasmic pH, its solubility in the lipid phase of the cell membrane would be very low; thus, inhibition of ATCase and pyrimidine synthesis would be prolonged.

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