Effects of lipids on nucleotide inhibition of wheat-germ aspartate transcarbamoylase: evidence of an additional level of control?

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Wheat-germ aspartate transcarbamoylase, a monofunctional trimer, is strongly inhibited by uridine 5'-monophosphate (UMP), which shows kinetic interactions with the substrate, carbamoyl phosphate, suggesting a classical allosteric mechanism of regulation. Inhibition of the purified enzyme by UMP was amplified in the presence of a variety of ionic lipids at concentrations low enough to preclude denaturation. In the absence of UMP, most of these compounds had no kinetic effect or were slightly activating. Two phospholipids did not show the effect. In a homologous series of fatty acids (C_6 – C_{16}), the potentiating

effect was only seen with homologues greater than C_8 , reaching a maximum at C_{12} . The effect of dodecanoate (C_{12}) on kinetic cooperativity (UMP as variable ligand) was studied. At each of several fixed concentrations of carbamoyl phosphate, dodecanoate had a pronounced effect on the half-saturating concentration of UMP, which was reduced by about half in every case, indicating substantially tighter binding of UMP. However, dodecanoate had relatively little effect on the kinetic Hill coefficient for the cooperativity of UMP. The possible metabolic significance of these effects is discussed.

INTRODUCTION

The mechanisms by which the sizes of pyrimidine nucleotide pools are regulated in higher plants are not as well understood as they are in micro-organisms. As one likely aspect of such regulation, the second enzyme in the pathway of *de novo* biosynthesis of pyrimidine nucleotides, aspartate transcarbamoylase (ATCase), has attracted interest largely as a consequence of the well-characterized role it plays in *Escherichia coli*, whose ATCase is now among the best-characterized of allosteric enzymes regulated by feedback inhibition. In higher plants, most (but not all) studies on the subcellular location of the ATCase point to the chloroplasts or plastids [1–3], although the protein is synthesized on cytoplasmic ribosomes [4]. Several workers have cloned and sequenced plant ATCase genes [5–8]. The complete ATCase sequences show typical chloroplast leader sequences [6]. The sequences, supported by immunochemical and other evidence [2,7], confirm that, unlike fungal and animal ATCases, the ATCase in plants is not covalently fused to the preceding or ensuing enzymes in the pathway, i.e. carbamoyl phosphate synthetase (CPSase) or dihydro-orotase.

Plant ATCases have been shown to be strongly inhibited by UMP *in vitro*, indicating regulation by a typical feedback loop [9–11]. Endogenously, UMP was initially shown to inhibit either CPSase or ATCase [12], and was eventually confirmed as inhibiting ATCase [13]. Williamson and Slocum [6] have tentatively identified a UMP-binding site on the catalytic chain sequence, judged by similarity with the CTP-binding site on the regulatory chain of the *E. coli* ATCase. Kinetic studies on the inhibition by UMP of ATCase from wheat germ suggested a classic 'Monod–Wyman–Changeux (MWC)' [14] two-conformation allosteric mechanism of regulation with carbamoyl phosphate as the antagonistic substrate [15]. The possibility that non-nucleotide metabolites may modulate the UMP-inhibition of

plant ATCases was first suggested when deoxycholate was shown to intensify the inhibition by UMP *in vitro* [16]. The serendipitous finding that 11-aminoundecanoate immobilized on Sepharose is an excellent affinity matrix for purifying the enzyme [17] indicated the presence of binding sites for lipophilic compounds and again raised the possibility of endogenous lipophilic effectors. A low-molecular-mass fraction from wheat germ was shown to include lipid-like compounds capable of intensifying UMP inhibition of the wheat-germ ATCase (R. J. Yon and A. Khan, unpublished work). In the present paper we report the results of an investigation into the effects of various water-soluble lipids on the regulatory behaviour of the ATCase purified from wheat germ, and discuss the possible metabolic significance of these effects.

MATERIALS AND METHODS

Materials

ATCase was purified from wheat germ by a revised, scaled up procedure (R. J. Yon and A. Khan, unpublished work; based on ref. [17]). The essential changes involved: (a) a new synthesis of the affinity adsorbent, 10-carboxydecyl-Sepharose; (b) steps to reduce the extraction of fine particulates and DNA from wheat germ; (c) omission of the heating step; (d) removal of aromatics by charcoal; and (e) configuring the affinity-chromatography stage for a 10-fold increase in scale. The purified enzyme was shown to be more than 95% pure by SDS- and non-denaturing gel electrophoresis. It was stored at a concentration of about 10 μ M in 25% glycerol at 4 °C. Under these conditions the enzyme retains the properties it has as a fresh extract for at least 6 months. Shortly before use, the enzyme was diluted 100-fold into 0.1 M Tris/HCl, pH 8.5. Its final dilution in the reaction mixture (see below) was 1500-fold, an operating enzyme con-

Abbreviations used: ATCase, aspartate transcarbamoylase; CPSase, carbamoyl phosphate synthetase; CMC, critical micelle concentration; MWC, Monod–Wyman–Changeux.

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Figure 1 Effects of even-chain fatty acids on initial-rate activity in the absence (■) and presence of UMP (●, 50 μ M; ◆, 100 μ M)

Activities are expressed as percentages of the activity in the absence of both lipid and UMP.

centration of approximately 1 nM. At this concentration the enzyme is stable for at least 1.5 h.

Carbamoyl phosphate, L-aspartate, UMP and fatty acids (as sodium salts) were obtained from Sigma. Buffer components were either from Sigma or from BDH.

Enzyme assay

The reaction mix (total volume 1.5 ml) contained diluted enzyme, buffer (0.1 M Tris/HCl, pH 8.5), L-aspartate (1 mM), carbamoyl phosphate (0.2, 1 or 5 mM) plus UMP and test lipid at various concentrations. The most stable components (i.e. excluding carbamoyl phosphate and enzyme) were mixed first, and carbamoyl phosphate was added last to start the reaction. Reaction was at 25 °C for 20 min; prior tests ensured that the reaction was linear (extent of reaction less than 10%) in this time. The reaction was stopped by adding 1.0 ml of reagent A (antipyrine/ H_2SO_4) of Prescott and Jones [18], followed by 0.5 ml of reagent B (butanedione monoxime/acetic acid). The concentration of the product, carbamoyl aspartate, was determined by the overnight method of Prescott and Jones [18]. Carbamoyl aspartate standards and appropriate blanks were included routinely.

Data handling

For inverted hyperbolic curves (see Figure 1) the values of the reaction rate at saturation by lipid ($V_{\text{sat.}}$) and half-saturating

ligand concentration ($K_{\text{app.}}$) were obtained by an adaptation of the non-linear fit to the Michaelis–Menten equation using the Wilkinson method [19]. $V_{\text{sat.}}$ was used to obtain a ‘potentiating factor’ defined as follows:

Potentiating factor =

$$\frac{\% \text{ inhibition by } 50 \mu\text{M UMP at lipids atn.}}{\% \text{ inhibition by } 50 \mu\text{M UMP in absence of lipid}} \quad (1)$$

Sigmoidal curves were first transformed to the appropriate Hill plot whose gradient (Hill coefficient, h_{UMP}) and intercept were found by linear regression. The half-saturating UMP concentration ($K_{0.5}$) was calculated from the intercept.

RESULTS

Effects of fatty acids and mild detergents on inhibition by UMP

The effects of several lipid compounds on enzyme activity in the absence of UMP, and in the presence of 50 and 100 μ M UMP were examined; in all cases the substrate concentrations were 1.0 mM for both L-aspartate and carbamoyl phosphate.

Since they are among the lipid compounds most likely to affect the enzyme *in vivo* (see the Discussion), fatty acids were examined in some detail. Figure 1 shows the effects of increasing concentrations of fatty acid salts of even C-length from C_6 to C_{16} . In the

Table 1 Apparent affinities (K_{app}) and potentiating factors of various ionic lipids

Inhibition curves (Figure 1 and similar results) were treated as inverted hyperbolas, and the parameters K_{app} and V_{sat} were derived as described in the Data handling section. Data in the presence of 50 μ M UMP were used. V_{sat} was used to obtain the potentiating factor as described; Figures in parentheses were obtained from V_{sat} values with standard errors greater than 50% of the mean. Abbreviation: N.D., not determined.

Compound	K_{app} (μ M)	Potentiating factor
Fatty acids		
Octanoate	218 $\pm$ 145	(1.85)
Decanoate	84 $\pm$ 18	3.07
Dodecanoate	12.3 $\pm$ 3.1	2.19
Tetradecanoate	21.5 $\pm$ 12.4	(3.00)
Hexadecanoate	33.9 $\pm$ 22.0	ND
Other lipids		
Dodecyl sulphate	8.0 $\pm$ 1.2	2.55
Decyl sulphonate	17.6 $\pm$ 6.6	1.86
Deoxycholate	124 $\pm$ 69	(2.21)
11-Aminoundecanoate	620 $\pm$ 230	(2.33)
Decyltrimethylammonium bromide	424 $\pm$ 131	(1.90)

absence of UMP, most fatty acid salts either had no effect, or had slightly activating effects (Figure 1, $\blacksquare$). In the case of tetradecanoate, activation peaked at around 30 μ M. Hexadecanoate was the only fatty acid tested that was an inhibitor in its own right, i.e. showed inhibition in the absence of UMP. The nature of this inhibition has not been studied in detail.

The most interesting effects of fatty acids occurred in the presence of UMP. Fatty acid salts with chains of C_8 or longer had a potentiating effect on UMP inhibition (Figure 1; $\bullet$, $\blacklozenge$). For a given, fixed UMP concentration, the fatty acid effect was hyperbolic, permitting in each case an estimate of the putative dissociation constant for the fatty acid (concentration at half-maximal change in inhibition, designated K_{app}) and a potentiating factor, using essentially the same statistics as for Michaelis–Menten kinetics (see Data-handling section above). The parameter K_{app} was strongly dependent on hydrocarbon chain-length (see Table 1), reaching its lowest value (tightest binding) at a chain-length of C_{12} . The value of K_{app} at C_{16} must be viewed cautiously since the apparent hyperbolic curves (in the presence of UMP) are complicated by the inhibiting effect of the fatty acid in its own right (see Figure 1). Potentiating factors (defined under Data handling, above) were generally in the range 2–3 with no single fatty acid significantly different from the rest (Table 1). Although critical micelle concentrations (CMCs) for fatty acids under the specific conditions of these experiments have not been reported, CMCs obtained under reasonably similar conditions [20] suggest that in all of these cases the enzyme was interacting with single molecules of fatty acid rather than with micelles.

These potentiating effects on UMP inhibition were also observed when a number of other amphipathic compounds were tested at concentrations low enough to avoid inactivation by detergent effects. These are summarized in Table 1. Strong potentiators included dodecylsulphate and decylsulphonate. Weaker potentiators included deoxycholate, 11-aminoundecanoate and dodecyltrimethylammonium bromide. No potentiation was observed for two long-chain phospholipids, dipalmitoylphosphatidylserine and dipalmitoylphosphatidylcholine. The apparent weak binding of 11-aminoundecanoate is somewhat surprising, since this is the ligand that was found (serendipitously)

Figure 2 Effects of dodecanoate on UMP cooperativity at 1 mM carbamoyl phosphate

(A) Activity as a function of UMP concentration, in the absence of dodecanoate ($\blacksquare$) and in the presence of 20 μ M ($\bullet$) and 50 μ M ($\blacklozenge$) dodecanoate. All activities are expressed as percentages of the activity in the absence of UMP. (B) The same data as Hill plots.

to be an excellent affinity adsorbent for the enzyme when it is immobilized on Sepharose [17].

Effects of dodecanoate (C_{12}) on co-operativity of UMP

With the concentration of substrates held constant, initial-rate activity varies with UMP concentration in a distinctly sigmoidal fashion, which has been interpreted as evidence of co-operativity between UMP-binding sites [15,21]. We examined the effect of fatty acid concentration on this cooperativity, using dodecanoate as a representative fatty acid.

Figure 2 shows initial-rate data at 0, 20 and 50 μ M dodecanoate, with carbamoyl phosphate constant at 1.0 mM. The data are plotted directly in Figure 2(A) and in the Hill plot format in Figure 2(B). Displacement of the curves parallel to the [UMP] axis in Figure 2(A) shows that increasing the concentration of dodecanoate significantly decreases the concentration of UMP giving half-maximal inhibition ($K_{0.5}$), from 88 μ M UMP (no dodecanoate) to 31 μ M UMP (50 μ M dodecanoate). This signifies a marked increase in the enzyme's affinity for UMP when dodecanoate is present. However, the co-

Figure 3 Effect of dodecanoate on UMP cooperativity at various carbamoyl phosphate concentrations

Measurements were taken at 0.2 (■), 1.0 (●) and 5.0 (◆) mM carbamoyl phosphate. In each case an appropriate range of UMP concentrations was selected to cover the middle (linear) region of the Hill plot, which was analysed as described above (see the Data handling section). Note logarithmic scale of ordinate.

operativity between UMP sites, measured by the Hill coefficient, h_{UMP} (gradient of the Hill plot), is relatively unaffected by dodecanoate, as shown by the almost parallel plots in Figure 2(B), where the slope differences are within the range of experimental error.

The effects of dodecanoate were also examined at a lower (0.2 mM) and a higher (5 mM) concentration of the antagonistic substrate, carbamoyl phosphate. As expected from the proposed MWC allosteric mechanism, changing the substrate concentration produces corresponding changes in the $K_{0.5}$ value in the absence of dodecanoate [15,21]. Apart from this expected change, the effects of dodecanoate are very similar at each substrate concentration, i.e. $K_{0.5}$ is roughly halved by 50 μM dodecanoate (shown in Figure 3), while h_{UMP} remains constant at about 2. Thus the effects of dodecanoate appear to be independent of the concentration of carbamoyl phosphate.

DISCUSSION

Synergistic feedback inhibition of wheat-germ ATCase *in vitro*

It has long been known [16] that, *in vitro*, UMP is a more potent inhibitor of wheat-germ ATCase in the presence of deoxycholate than in its absence. We can now comment on the specificity of this potentiating effect, judged by the apparent binding constant (K_{app}) of various lipids. The effect is shown optimally by long-chain saturated anionic lipids (C_{10} – C_{14}), whether carboxylates, sulphates or sulphonates. Cationic (e.g. decyltrimethylammonium bromide) and zwitterionic (e.g. 11-aminoundecanoate) lipids are only effective at considerably higher concentrations, as are cholates (such as deoxycholate) with more rigid hydrophobic moieties. These findings all suggest that a 12-carbon, flexible hydrophobic chain attached to a negative ion provides the optimum structure. This structure acts by increasing the enzyme's affinity for UMP, but appears to have little or no effect on the mechanism of the allosteric interaction between UMP and carbamoyl phosphate (at least in so far as it affects the Hill coefficient). On the limited evidence of two samples, phospholipids appear to be ineffective. The effects of anionic

lipids with unsaturated chains or aromatic ring structures have not been examined.

In addition to the synergistic (potentiating) effect of medium-chain-length (C_{10} – C_{14}) fatty acids, C_{16} (and presumably longer) fatty acids are inhibitors of ATCase in their own right (see Figure 1), with quite strong binding (dissociation) constants in the region 50–100 μM .

Metabolic significance

Since we have only studied the above effects *in vitro*, we turn next to consider whether they have any possible metabolic significance. It is known that the low-molecular-mass fraction of a water-soluble extract of wheat germ (essentially the embryos of dormant, dehydrated wheat seeds) contains lipid compounds capable of potentiating UMP inhibition, although the fraction has not been fully analysed (R. J. Yon and A. Khan, unpublished work). These compounds, like the enzyme, were obviously produced in the metabolically active stages of seed development that preceded dormancy. On the basis of the specificity discussed above, one can speculate on some of the likely candidates for potentiating lipids *in vivo*. There is a considerable variety of anionic lipids in plant seeds [22] most of which have not been tested, and they include several (e.g. long-chain prenol phosphates) which have structures close to the optimal structure discussed above. While recognizing these facts, our present thinking favours free fatty acids, for reasons which will emerge below.

We can rationalize the effects reported in this paper at two metabolic levels. (1) It would not be surprising to find that, in times of rapid cell growth and cell division, plants have devised strategies to co-ordinate the biosynthesis and breakdown of some of their major biochemical requirements, i.e. membranes, RNA and protein. One can view the present effects as part of a broad co-ordination of RNA and membrane biosynthesis, at the levels of provision of pyrimidine nucleotides and fatty-acyl groups respectively. (2) In seed development, a more specific connection between the biosynthesis of fatty acids and of pyrimidine nucleotides can be discerned. A significant part of the fatty acid production in many plant seeds is directed to storage oils (triacylglycerols) via CDP-diacylglycerol (reviewed in [23]). When oil production moves into excess (signalled by an excess of fatty acids), the synthesis of CDP as well as of fatty acids may have to be feedback-inhibited. In both of these scenarios, the use of medium-chain fatty acids to intensify the feedback inhibition of ATCase by UMP, and of long-chain fatty acids to inhibit ATCase directly, would make sense. In both scenarios too, fatty acids would be the lipids favoured to regulate ATCase inhibition *in vivo*.

In further support of fatty acids, it should be noted that ATCase and fatty acids are similarly compartmentalized within the plant cell. There is an impressive body of evidence indicating that the mature plant ATCase is located in chloroplasts or plastids ([1–3]; A. Aibangbee, R. Miller and R. Yon, unpublished work). Fatty acid synthesis is similarly localized, although acyl carrier proteins rather than free fatty acids are involved. However, some acylated acyl carrier proteins are hydrolysed by soluble thioesterases to form free fatty acids inside the stroma [24]. These fatty acids would be destined for export from the chloroplast for glycerolipid synthesis [23], i.e. they are precisely the pool of fatty acids that would be expected to regulate the ATCase, if our second speculation (above) is correct.

Why the potentiating effect should be focused on medium-chain (around C_{12}) fatty acids, rather than C_{16} and longer fatty acids (the major products of fatty acid biosynthesis) remains an

open question at present. It is of interest to note, in this context, that some recent reports suggest interesting new roles for medium-chain fatty acids in plant metabolism and its regulation (see, for example, [25,26]).

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