Aurintricarboxylic acid is a potent inhibitor of phosphofructokinase

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Aurintricarboxylic acid (ATA) was found to be a very potent inhibitor of purified rabbit liver phosphofructokinase (PFK), giving 50 % inhibition at 0.2 μ M. The inhibition was in a manner consistent with interaction at the citrate-inhibitory site of the enzyme. The data suggest that inhibition of PFK by ATA was not due to denaturation of the enzyme or the irreversible binding of inhibitor, since the inhibition could be reversed by addition of allosteric activators of PFK, i.e. fructose 2,6-bisphosphate or AMP. Two other tricarboxylic acids, agaric acid and (–)-hydroxycitrate, were found to inhibit PFK. ATA at much higher concentrations (500 μ M) was shown to inhibit fatty acid synthesis from endogenous glycogen in rat hepatocytes; however, protein synthesis was not altered.

INTRODUCTION

Aurintricarboxylic acid (ATA) has been used extensively as an inhibitor of protein synthesis in cell-free systems [1,2] and more recently to stimulate transcription and translation in mitochondrial lysates [3]; however, the effect of ATA on other enzymes has not been reported. Phosphofructokinase (PFK) activity has been observed to be inhibited by polycarboxylic aromatic compounds as well as by various citrate analogues [4]. Since ATA is a carboxylic acid, it was possible that ATA might also inhibit PFK. In the present study the effect of ATA on purified PFK has been examined.

Previous studies with isolated hepatocytes in this laboratory have demonstrated that some other carboxylic acids inhibit lipogenesis [5–7]; therefore a preliminary study on ATA's effect on lipogenesis and protein synthesis is reported.

MATERIALS AND METHODS

Enzyme purification and assays

PFK was purified from rabbit liver as described by Kemp [8], with minor modifications. In the modified procedure, the $(\text{NH}_4)_2\text{SO}_4$ cut was replaced by precipitation with 6 % (w/v) polyethylene glycol 6000, the ion-exchange step was performed with DEAE-Sephadex A-25, and the gel filtration was performed on Sephacryl S-300. The enzyme was over 90 % homogeneous by polyacrylamide-gel electrophoresis. PFK was assayed as described previously [9], except that the pH was 7.3. Glycerol-phosphate dehydrogenase was assayed in 50 mM-Tes/NaOH, pH 7.3, containing 150 mM-KCl, 1 mM-EDTA, 1 mM-dithiothreitol, 0.2 mM-NADH and 0.2 mM-dihydroxyacetone phosphate. Aldolase was assayed in the same solution, except that 0.1 mM-fructose 1,6-bisphosphate replaced dihydroxyacetone phosphate and the solution contained 26 μ g of glycerol-phosphate dehydrogenase and 0.5 μ g of triose-phosphate isomerase per ml.

Pyruvate kinase was assayed in 50 mM-Tes/NaOH, pH 7.3, containing KCl, EDTA, dithiothreitol and NADH as above, with the addition of 5 mM-MgCl₂, 0.5 mM-ADP, 0.5 mM-phosphoenolpyruvate and 20 μ g of lactate dehydrogenase/ml of solution. Lactate dehydrogenase was assayed similarly, where the substrate was 0.2 mM-pyruvate and the ADP and phosphoenolpyruvate were omitted. Fructose-bisphosphatase was assayed as described by Gottschalk *et al.* [10], with 0.1 mM-fructose 1,6-bisphosphate as substrate. When testing the above enzymes for inhibition by ATA, approx. 20 m-units of enzyme/ml of assay solution were used. ATP citrate lyase was assayed as described by Roehrig *et al.* [11].

Hepatocyte studies

Hepatocytes were prepared by the method of Berry & Friend [12] with minor modifications [13], between 09:00 and 10:00 h from 200–300 g male Sprague–Dawley rats either fed *ad libitum* on Purina Rat Chow (06:00–18.00 h light cycle) or starved for 48 h. Cells (50–60 mg wet wt.) were suspended in 2 ml of Krebs–Henseleit buffer supplemented with 2.5 % (w/v) bovine serum albumin under an atmosphere of O₂/CO₂ (19:1) in stoppered 25 ml Erlenmeyer flasks. Incubations, in a shaking water bath at 37 °C, were terminated with HClO₄. The rate of fatty acid synthesis, expressed as μ mol of acetate equivalents/min per g wet wt. of hepatocytes, was determined by the incorporation of ³H₂O into total lipid fatty acids [13]. The rate of protein synthesis was determined by incorporation of [³H]leucine as described previously [14].

Materials

ATA, agaric acid and all biochemical reagents were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase was obtained from Worthington Biochemical Corp., Malvern, PA, U.S.A. Radioisotopes were purchased from Research Products International, Mount Prospect, IL, U.S.A. (–)-Hydroxycitrate was

Abbreviations used: ATA, aurintricarboxylic acid; PFK, phosphofructokinase.

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RESULTS AND DISCUSSION

It was found that ATA is a potent inhibitor of liver PFK *in vitro*. As shown in Fig. 1, PFK was inhibited by 50% by 0.2 μM -ATA at pH 7.3 under the conditions used. The enzyme was virtually unaffected by 2.0 μM -ATA when assayed at pH 8, where PFK does not exhibit allosteric behaviour. As shown in Fig. 2, ATA increased both the $s_{0.5}$ for fructose 6-phosphate and the sensitivity to ATP inhibition. Fructose 2,6-bisphosphate counteracted this inhibition (Fig. 2). In a separate experiment (Table 1), the data suggest that inhibition of PFK by ATA was not due to denaturation of the enzyme or the irreversible binding of inhibitor. Not only did inhibition tend to be reversed by addition of allosteric activators of PFK, but PFK could be incubated with up to 10 μM -

Fig. 1. Inhibition of PFK by ATA

PFK was assayed at pH 8.0 (●) or at pH 7.3 (○) as described in the Materials and methods section. The concentrations of ATP and fructose 6-phosphate were 0.5 and 1.0 mM respectively. The concentration of ATA was calculated on the basis of its nominal M_r of 473 for the monomeric form, although the compound probably exists as a heterogeneous collection of polymers [15].

ATA for 30 min with no loss of activity at pH 8 (results not shown).

However, ATA proved at higher concentrations to be a general enzyme inhibitor. For example, the auxiliary enzymes of the PFK assay (aldolase and glycerol-phosphate dehydrogenase from rabbit muscle) were sensitive to higher concentrations of ATA, being inhibited by 50% by about 3 μM -ATA. The PFK assays were not affected, however, since these were performed at ATA concentrations no higher than 1 μM and in the presence of an excess of auxiliary-enzyme activity. Other enzymes also were inhibited by much higher concentrations of ATA. Pyruvate kinase and lactate dehydrogenase from rabbit muscle were inhibited by 50% by 50 μM - and 20 μM -ATA respectively. Rat liver fructose-1,6-bisphosphatase (supplied by Dr. Frank Marcus of Chicago Medical School) was inhibited by 50% by about 10 μM -ATA. ATP-citrate lyase was inhibited by 37% at 50 μM -ATA (assayed by Dr. K. Roehrig of Ohio State University).

Although we calculated the concentration of ATA on the basis of the M_r of its nominal tris(salicyl) methane structure, Gonzalez *et al.* [15] reported that ATA existed as a heterogeneous collection of polymers of this basic structure; those authors partially fractionated these polymers by dialysis and ultrafiltration. We accomplished a similar fractionation of ATA by passage over Sephadex G-50 equilibrated with 10 mM- NH_3 . ATA fractions eluted in the void volume inhibited PFK to the same extent on a weight basis as did fractions eluted in the salt volume after freeze-drying and reconstitution in water. Therefore it appeared that all polymeric forms of ATA were equally effective on a weight basis in inhibiting PFK. In addition, ATA could be desalted by passage over Sephadex G-15 equilibrated with 10 mM- NH_3 ; the G-15-treated ATA inhibited PFK equally as well as did unfractionated ATA, indicating that low- M_r impurities were not responsible for the inhibition.

Given that ATA is an allosteric inhibitor of PFK, the question of its exact site of action arises. We speculate that ATA may interact with the citrate-binding site on PFK, since inhibition of PFK by ATA is qualitatively similar to inhibition of PFK by citrate. Also, the citrate-binding site on PFK is known to be relatively non-

Fig. 2. Inhibition of PFK at pH 7.3 by ATA as a function of fructose 6-phosphate or ATP concentration

In (a), the concentration of ATP was 1 mM, with the addition of 1 μM -ATA (●), 3 μM -fructose 2,6-bisphosphate (○), 1 μM -ATA + 3 μM -fructose 2,6-bisphosphate (■), or no additions (□). In (b), the concentration of fructose 6-phosphate was 1 mM, with the addition of 0.2 μM -ATA (●), 0.5 μM -ATA (○), or no ATA (□).

Table 1. Reversal of ATA inhibition of PFK

PFK was assayed at pH 7.3 in the presence of 1 mM-ATP, 1 mM-fructose 6-phosphate, 1 μ M-ATA and additions as indicated. $V_{\max}$ was defined as the rate at 1 mM-ATP and 10 mM-fructose 6-phosphate. The activators were added last, after the reaction had been initiated. Abbreviation: Fru-2,6- P_2 , fructose 2,6-bisphosphate.

Addition	Rate (% of $V_{\max}$)
None	5
5 mM- P_i	23
0.1 mM-AMP	28
5 mM- P_i + 0.1 mM-AMP	40
3 μ M-Fru-2,6- P_2	58
3 μ M-Fru-2,6- P_2 + 0.1 mM-AMP	75
3 μ M-Fru-2,6- P_2 + 0.1 mM-AMP + 5 mM- P_i	85

specific, being able to accept various phosphoglycerates and phosphoenolpyruvate as well as citrate [16]. Passonneau & Lowry [4] even reported inhibition of PFK by polycarboxylic aromatic compounds as well as by various citrate analogues. However, the extremely high affinity of ATA for liver PFK, of the order of 10^3 times the affinity of citrate for liver PFK, is unusual.

In hepatocytes isolated from fed male rats, the inhibition of fatty acid synthesis was significant at 500 μ M-ATA (control, 0.025 ± 0.003 μ mol/min per g wet wt.; + ATA, 0.019 ± 0.002). In a dose-response study (50–1000 μ M-ATA), minimal effects were observed at the lowest concentration, with inhibitions reaching their maximum near 500 μ M and higher concentrations showing only slightly more inhibition (results not shown). However, protein synthesis was not altered at any concentration of ATA (control, 0.0015 ± 0.0003 μ mol/min per g wet wt.; + 500 μ M-ATA, 0.0014 ± 0.0002). The concentration needed to produce inhibition of fatty acid synthesis in cells was high relative to the amount needed to inhibit purified enzymes *in vitro*. A similar situation has been observed with (–)-hydroxycitrate, another lipid-lowering tricarboxylic acid. (–)-Hydroxycitrate is a very potent inhibitor of purified ATP citrate lyase [17], but requires a much higher concentration to inhibit lipid synthesis in isolated hepatocytes [18,19]. This may be a common problem with tricarboxylic acids in hepatocytes.

Two other tricarboxylic acid which decrease fatty acid synthesis in hepatocytes, agaric acid [18] and (–)-hydroxycitrate [19], were also tested to see if they inhibit PFK. In our assay system PFK was inhibited by 73 % by 10 μ M-agaric acid, 50 % by 1 mM-(–)-hydroxycitrate and 50 % by 1 mM-citrate. In hepatocytes incubated with agaric acid (60 μ M) a significant unexplained fall in lactate concentration was observed at the 30 min incubation

time [16]. This might be explained by inhibition of PFK in the cell. These findings might be due to a change in redox state because of repression of glycolysis. Another explanation might be that one of the many other sites of inhibition proposed for agaric acid (citrate transport, adenine nucleotide transport and the enzyme aconitase [18]) might cause these effects on lactate. With the current studies we have not ruled the possibility that ATA at higher concentrations or with longer periods of incubation might be inhibiting other enzymes or transporters in the cells.

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