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Phosphorus-containing inhibitors of aspartate transcarbamoylase from *Escherichia coli*

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A tetrahedral intermediate is the prominent feature of the generally accepted mechanism for aspartate transcarbamoylase. We have synthesized *N*-pyrophosphoryl-L-aspartate as a charged analogue of the postulated intermediate. Surprisingly, its affinity for the enzyme from *Escherichia coli* was substantially lower than that of the previously known inhibitor phosphonoacetyl-L-aspartate which contained a trigonal carbonyl group. Similar results were obtained with the corresponding mercaptosuccinate derivatives. We also tested a number of new pyrophosphate analogues as inhibitors. Our results cast doubt on some aspects of the current model for the mechanism of this enzyme.

Enzyme inhibition; Transition state; *N*-Pyrophosphoryl-L-aspartate; Aspartate transcarbamoylase; (*Escherichia coli*)

1. INTRODUCTION

ATCase (EC 2.1.3.2) has aroused considerable interest both as a model regulatory enzyme [1] and as a potential target for chemotherapy [2]. In a previous attempt to develop tight-binding inhibitors, we probed the active site of the enzyme from *Escherichia coli* with various substrate analogues [3]. The results suggested that stabilization of a negatively charged transition state might be an important aspect of the catalytic mechanism. We have now tested the hypothesis by synthesizing a putative transition-state analogue. The binding of this inhibitor and that of other phosphorus-containing compounds provide additional insight into the structure and function of this enzyme.

2. MATERIALS AND METHODS

ATCase was isolated from *E. coli* and the subunits were separated by well-established methods [4,5]. Enzyme activity was measured following incorporation of ^{14}C label from carbamoyl phosphate as previously described [3] except that sodium carbonate (100 μl of 0.1 M) was added to each vial instead of crushed dry ice to complete the removal of $^{14}CO_2$. The pattern of inhibition was determined by analyzing the data with double reciprocal plots [6] and the inhibition constants were estimated according to Dixon [7]. The K_i values are average values of at least two separate determinations using four different concentrations of both carbamoyl phosphate and inhibitor.

All starting materials were used without further purification and were obtained from Sigma Chemical, except for phosphorus oxychloride and triethylamine (BDH Chemicals), D,L-mercaptosuccinic acid and thionyl chloride (Aldrich Chemicals), acetonitrile (Fisher Scientific), and 3-phosphonopropionic acid (Fluka AG). Peroxydiphosphonate was a gift from Prof. N.J. Leonard, University of Illinois at Urbana-Champaign, USA.

The synthesis and characterization of dichloromethylene diphosphonate, carbonyl diphosphonate and hypophosphate have been reported elsewhere [8-10]. *N*-Ethylphosphonoformamide was prepared analogously to what has been described for other carbamoyl phophonic acids [11].

N-pyrophosphoryl-L-aspartate was prepared by the reaction of pyrophosphoryl tetrachloride with L-aspartate under conditions previously used for the synthesis of *N*-phosphoryl-L-aspartate [12]. The mercaptosuccinate derivatives were synthesized by treatment of the thiol with the acid chloride in acetonitrile in the presence of triethylamine. The products were isolated by column chromatography or fractional precipitation [12]. The methods used to ascertain purity included ^{31}P-NMR and quantitative analysis before and after hydrolysis. The analytical procedures covered the determination of aspartate [3], phosphate and pyrophosphate [13], and free thiol and thiol ester content [14]. Details of the synthesis and characterization of these new inhibitors will be published separately (Laing, N. and Chan, W.W.-C., in preparation).

3. RESULTS AND DISCUSSION

The reaction catalyzed by ATCase is generally considered to proceed via a nucleophilic attack by the free amino group of L-aspartate on the carbon of carbamoyl phosphate [15,16] (fig.1). The ensuing tetrahedral intermediate may either accept a proton or remain as a fully charged oxyanion. Since previous results appeared to favor the latter possibility [3], we deemed it worthwhile to synthesize *N*-pyrophosphoryl-L-aspartate (fig.1) as a potential transition-state analogue. As in our earlier study [3], the effects of various compounds were tested on the catalytic subunit

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Abbreviations: ATCase, aspartate transcarbamoylase; PALA, *n*-phosphonoacetyl-L-aspartate

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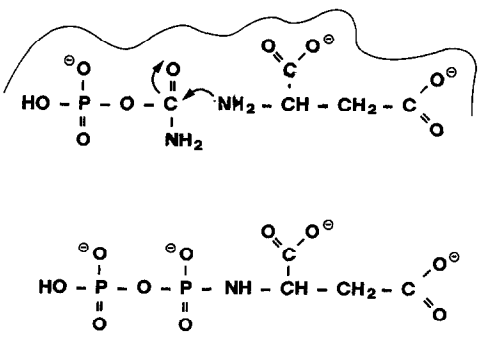

Fig. 1. Schematic representation of the active site of ATCase. The top section illustrates the generally accepted mechanism of the reaction. The lower section shows the structure of the putative transition-state analogue aligned in the same manner as the substrates.

of the enzyme from *E. coli*, all of which inhibited the enzyme competitively with respect to carbamoyl phosphate.

Although *N*-pyrophosphoryl-L-aspartate inhibits ATCase, its affinity is much weaker than expected (see **2** in table 1). In fact, it binds to the enzyme approximately 9-fold less tightly than the bisubstrate analogue PALA (**1**), discovered many years ago by Collins and Stark [17]. This surprising result indicates that binding occurs preferentially with an uncharged trigonal carbonyl group in this critical position of the inhibitor. Nevertheless, the pyrophosphoryl group must interact with the enzyme favorably overall since the corresponding phosphoryl derivative (**3**) has a 200-fold lower affinity.

In order to confirm these observations, we also synthesized the sulfur analogues of the above aspartate derivatives. The behavior of this series of compounds is qualitatively similar to that of their nitrogen-containing counterparts. Thus *S*-phosphonoacetyl-mercaptosuccinate (**4**) is a significantly more powerful inhibitor than *S*-pyrophosphoryl-mercaptosuccinate (**5**), which is in turn stronger than the *S*-phosphoryl derivative (**6**). The differences in affinity between these inhibitors are, however, much smaller than those in the aspartate series, suggesting that the NH group may have a special role in promoting tight binding of adjacent functional groups. It is also clear that the NH group makes a large contribution towards the strength of binding. Thus the affinity of PALA for ATCase is at least 100-fold higher than that of its sulfur analogue (even allowing for the possibility that only the L-isomer of the latter may be active). It is interesting that the oxygen analogue (**7**) synthesized by Farrington et al. [18] has an affinity similar to that of the sulfur derivative. This result may mean that the hydrogen bonding capability of the NH group is responsible for the stronger interaction of the aspartate derivatives.

The above observations seemed at first to be incompatible with the previous finding of an anion-binding site at the reaction centre. We therefore proceeded to investigate more fully the binding of phosphate and phosphonate derivatives containing additional negative charges. Pyrophosphate (**9**, table 2) may be regarded as the classical example of this type of compound and it has already been shown to be a good inhibitor. We find that the distance between the phosphate groups has an important influence on the affinity of these derivatives. Thus hypophosphate (**8**) binds significantly tighter than pyrophosphate, whereas peroxydiphosphonate (**10**) is a much weaker inhibitor. A similar effect has also been demonstrated for phosphonoformate (**11**) and phosphonoacetate (**12**) [3]. A further increase in distance between the charged groups (**13**) led to an even greater decrease in affinity. It is noteworthy that hypophosphate and phosphonoformate have virtually identical inhibition constants for ATCase. Thus there seems to

Table 1

Inhibition of ATCase by dicarboxylate derivatives

	Inhibitor (R = $-CH(CH_2-CO_2^-)-CO_2^-$)	K_i (μM)
1	$^-O-P(=O)(-CH_2-C(=O)-NH-R)-O^-$ (L)	0.027 [17]
2	$^-O-P(=O)(-O-P(=O)(-NH-R)-O^-)-O^-$ (L)	0.24
3	$^-O-P(=O)(-NH-R)-O^-$ (L)	50 [3]
4	$^-O-P(=O)(-CH_2-C(=O)-S-R)-O^-$ (L)	5.5
5	$^-O-P(=O)(-O-P(=O)(-S-R)-O^-)-O^-$ (DL)	17
6	$^-O-P(=O)(-S-R)-O^-$ (DL)	125
7	$^-O-P(=O)(-CH_2-C(=O)-O-R)-O^-$ (L)	2.0 [18]

Table 2

Inhibition of ATCase by pyrophosphate analogues

Inhibitor	K_i (μM)	
8 $^{2-}O_3P-PO_3H^-$	20	
9 $^{2-}O_3P-O-PO_3H^-$	51	[21,3]
10 $^{2-}O_3P-O-O-PO_3H^-$	175	
11 $^{2-}O_3P-CO_2^-$	22	[3]
12 $^{2-}O_3P-CH_2-CO_2^-$	150	[21,3]
13 $^{2-}O_3P-CH_2-CH_2-CO_2^-$	7500	
14 $^{2-}O_3P-CH_2-PO_3H^-$	37	[3]
15 $^{2-}O_3P-NH-PO_3H^-$	71	
16 $^{2-}O_3P-CCl_2-PO_3H^-$	32	
17 $^{2-}O_3P-C(=O)-PO_3H^-$	3.5	
18 $^{2-}O_3P-C(=O)-NH-CH_2-CH_3$	2000	

be no preference for a particular configuration for this group as long as its charge and location are appropriate.

It had also been observed previously [3] that very little change in affinity occurred when the oxygen bridge of pyrophosphate was replaced by a methylene equivalent (**14**). Our results here also show that binding is not greatly affected when an imido (**15**) or a dichloromethylene group (**16**) occupies this bridge position. However, substitution by a carbonyl group at this site (**17**) leads to a highly potent inhibitor. The large increase in affinity in this case is probably due to the orientation of the phosphonate groups brought upon by the trigonal carbon atom rather than the interaction of the carbonyl group itself. This interpretation is supported by the finding that replacement of one phosphonate group by an ethylamido moiety (**18**) produced a drastic loss of affinity.

The evidence presented above supports the previous contention [3] that there is an anion-binding site in ATCase in the vicinity of the phosphate-binding region. However, it now appears that this site is not involved in stabilizing a negatively charged tetrahedral transition state as has been postulated. It is perhaps important to note that phosphoroamidates are potent inhibitors of some proteases whose reactions are believed to proceed via similar tetrahedral intermediates [19]. Thus it seems unlikely that the failure of *N*-pyrophosphoryl aspartate to act as a transition-state analogue in this work is due to the different properties (e.g. bondlengths) of phosphorus analogues. It should also be pointed out that in a previous study of ATCase [20], a neutral tetrahedral analogue incorporating a hydroxyl function was found to be a poor inhibitor compared to its trigonal counterpart (the corresponding ketone). We are therefore forced to consider the possibility that the mechanism of ATCase may not involve formation of a tetrahedral intermediate. A viable alternative would be for the reaction to proceed in a concerted manner in which nucleophilic attack by the amino group and departure of phosphate occur simultaneously. In such a mechanism, the anion-binding site may have the function of facilitating the cleavage of the phosphate group. It would seem worthwhile to design experiments for testing such a hypothesis.

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