Notes

Oxyanion Specificity of Aspartate-β-semialdehyde Dehydrogenase

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Introduction

L-Aspartate-β-semialdehyde dehydrogenase (ASADH) catalyzes the reversible reductive dephosphorylation of L-β-aspartyl phosphate to form L-aspartate-β-semialdehyde (ASA) utilizing NADPH. ASADH is located in the aspartate pathway of amino acid synthesis and catalyzes a key branch point reaction, with one branch leading to the biosynthesis of L-lysine and the other to L-methionine, L-isoleucine, and L-threonine.[1] A cysteine is the active site nucleophile in ASADH that attacks L-β-aspartyl phosphate, forming an acyl enzyme and releasing phosphate.[2] In the reverse direction the phosphate nucleophile attacks the acyl enzyme formed by the oxidation of ASA, yielding L-β-aspartyl phosphate. The high-resolution structure of ASADH from *Escherichia coli* has recently been solved[3] and confirms the presence of several active site amino acids whose roles in substrate binding and catalysis had previously been suggested by site-directed mutagenesis studies.[4] An examination of the putative phosphate binding site reveals the presence of several arginines and a lysine that can potentially be involved in the binding of this anion.[5] In this report, the specificity of this anion binding site has been examined, with the aim of identifying alternative substrates that can substitute for phosphate and competitive inhibitors that can stabilize the acyl enzyme intermediate to allow structural characterization.

Experimental Section

Materials. The sodium or potassium salts of most of the anions examined were obtained from Fisher, Alfa, or Aldrich. Sodium tungstate and potassium bromate were purchased from Merck. Potassium permanganate was obtained from Baker Chemical, and sodium tetrafluoroborate was from Acros Organics. L-Aspartic-β-semialdehyde (ASA) was prepared by the ozonolysis of L-allylglycine according to the method of Black and Wright.[6] Aspartate-β-semialdehyde dehydrogenase was purified by previously published methods.[7] NADP and dithiothreitol (DTT) were obtained from U.S. Biochemical, and Ches buffer was obtained from Fisher Biotech.

Enzyme Assays. Enzyme assays were run on a Perkin-Elmer Lambda 1 spectrophotometer equipped with a circulating water bath and a constant-temperature cell holder. ASADH activity was determined in the reverse (oxidative) direction on the basis of the appearance of NADPH absorbance at 340 nm. Assays were run at 25 °C in a 1 mL total volume quartz cuvette containing 200 mM Ches buffer, pH 8.6, 1 mM EDTA, 1 mM DTT, 1 mM NADP (K_m = 0.19 mM),[4] 0.5 mM ASA (K_m = 0.17 mM),[4] and either 5 mM phosphate or varied concentrations of the different oxyanions (when possible from 0.5 to 5 times the K_m or K_i). The oxyanions that did not interact were examined at concentrations of at least 20 mM, except for permanganate (which resulted in oxidation) and chromate (which has strong absorption at 340 nm). Vanadate has been shown to interact with a variety of buffers, as well as with DTT and EDTA.[8] These interacting components were eliminated during the examination of vanadate, and the concentration range and pH were chosen to ensure that monomeric vanadate was the predominant species.[9] All reactions were initiated by the addition of 0.5 μg of ASADH to the assay cocktail.

Bond Length and Charge Density Calculations. Central atom to oxygen bond lengths for the anions were calculated by adding the effective ionic radii of the respective atoms, without corrections for covalency or electronegativity differences. These ionic radii do take coordination number, geometry, and oxidation states into account,[10] and the calculated bond lengths correspond quite closely to the measured bond lengths that are available for these anions.[11,12] Ab initio molecular orbital calculations were performed with the Gaussian 94 program[13] on a Unix workstation. Each anion was investigated at a restricted Hartree−Fock level by using the 3-21G* basis set. Charge densities were obtained after initial estimates were entered for the geometry, overall charge, bond lengths, and bond angles for each anion. Because of the increasing number of orbitals, these calculations are limited to elements in the first five periods of the periodic table, so charge density calculations were not conducted for tungstate and perrhenate.

Results and Discussion

Oxyanion Specificity. An extensive list of oxyanions has been examined in order to identify compounds that can interact with ASADH as phosphate analogues. Of those that were examined, the oxyanions listed in Table 1 have been found to interact with ASADH either as an alternative substrate or as a competitive inhibitor. The kinetic parameters for arsenate are comparable to those of phosphate, with a V_{max} that is about 70% that of phosphate and a K_m that is nearly a factor of 2 lower. The enzyme has a 20-fold lower Michaelis constant for the alternative substrate vanadate and a V_{max}/K_m that is 10-fold higher than that seen with phosphate. For the competitive inhibitors, the inhibition constants range from that of perrhenate, which is nearly a factor of 50 higher than the K_m for phosphate, to that of periodate, which is over a factor of 10 lower. The oxyanions that interact with ASADH all have tetrahedral coordination geometry. The central atom to oxygen bond lengths

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Table 1. Kinetic Parameters for Oxyanions Interacting with ASADH

oxyanion	V_{max} (min^{-1})	K_m (mM)	V_{max}/K_m (M^{-1} s^{-1})	K_i (mM)
phosphate	**710 ± 33**	**2.9 ± 0.5**	**4.1 × 10^3**	
arsenate	510 ± 37	1.6 ± 0.4	5.3 × 10^3	
vanadate	330 ± 9	0.14 ± 0.01	3.9 × 10^4	
periodate				0.22 ± 0.08
tellurate				11 ± 0.5
phosphonate				17 ± 3
tungstate				26 ± 3
perrhenate				140 ± 60

for these substrates and inhibitors are all somewhat longer than the phosphorus−oxygen bond length.

Many of the oxyanions that were examined neither support nor inhibit the oxidative production of β-aspartyl phosphate that is catalyzed by ASADH. The molecular geometries of these noninhibitory anions are primarily tetrahedral, although some trigonal planar (nitrate and carbonate) and trigonal bipyramidal (chlorate and bromate) anions were also tested. For these noninhibitory anions, the central atom to oxygen bond lengths ranged from those that are considerably shorter than the phosphorus−oxygen distances (nitrate, carbonate) to those that are comparable (chlorate, sulfate, permanganate) or considerably larger (molybdate). While all of the alternative substrates and competitive inhibitors have bond lengths to oxygen that cluster around 1.7−1.8 Å, several noninhibitory anions such as chlorate and selenate also have bond lengths that are between these values and the bond length in phosphate (Table 2). Clearly, molecular size and geometry are not, by themselves, the only determinants in the binding of the oxyanion phosphate by ASADH.

Table 2. Anion Bond Lengths and Charge Densities

anion[a]	bond length (Å)[b]	charge on central atom	charge on oxygen
carbonate (CO_3^{2-})	1.27	+0.878	−0.964
phosphate (HPO_4^{2-})	**1.52**	**+1.263**	**−0.952**
phosphonate (HPO_3^{2-})	*1.79*	*+1.012*	*−0.906*
arsenate ($HAsO_4^{2-}$)	**1.69**	**+1.125**	**−0.892**
tellurate (TeO_4^{2-})	*1.78*	*+1.543*	*−0.886*
vanadate (HVO_4^{2-})	**1.71**	**+1.145**	**−0.885**
sulfate (SO_4^{2-})	1.47	+1.444	−0.861
selenate (SeO_4^{2-})	1.63	+1.393	−0.848
chromate (CrO_4^{2-})	1.61	+1.240	−0.810
molybdate (MoO_4^{2-})	1.86	+1.201	−0.800
thiosulfate (SSO_3^{2-})	1.47[c]	+1.347	−0.799
chlorate (ClO_3^-)	1.47	+1.159	−0.723
periodate (IO_4^-)	*1.77*	*+1.876*	*−0.719*
bromate (BrO_3^-)	1.66	+1.101	−0.701
perchlorate (ClO_4^-)	1.43	+1.640	−0.660
permanganate (MnO_4^-)	1.60	+1.443	−0.610
nitrate (NO_3^-)	1.25	+0.643	−0.553
tetrafluoroborate (BF_4^-)	1.40	+1.099	−0.525

[a] Substrates of ASADH are shown in **bold** and inhibitors in *italics*. [b] Central atom to oxygen bond lengths were calculated by addition of the effective ionic radii.[10] [c] The sulfur−sulfur bond length is calculated to be 1.96 Å.

Anion Charge Distribution. Calculations were carried out to determine if the charge distribution on the peripheral oxygen atoms plays an essential role in determining which anions will interact with ASADH. Using Gaussian 94, the charge distribution was determined for each anion in the predominant ionization state that is present under the assay conditions. The results of these calculations are listed in Table 2, arranged in order from the greatest negative charge on oxygen. Phosphate, the physiological substrate for ASADH, has the most negatively charged oxygens among the tetrahedral anions that were examined. This substrate is followed closely by arsenate and vanadate, the alternative substrates that have been identified. Tellurate and periodate, both competitive inhibitors vs phosphate, also were found to have a high negative charge on oxygen.

It appears that the charge density on the oxygen atoms of the anion plays an important role in the selection of anion substrates. Carbonate has the greatest charge density on oxygen among the anions examined, but its planar geometry apparently prevents sufficient interactions with the anion binding groups of ASADH. Phosphate, with the most negative charge density on oxygen among the tetrahedral anions, is the physiological substrate for ASADH. The oxyanions with the next most negative charge densities on oxygen are either good alternative substrates or inhibitors for this enzyme. Thus, it appears that the binding groups in the anion site will preferentially interact with oxyanions that are both tetrahedral and have high negative charge density on the peripheral oxygens. The only anomaly is periodate, which is tetrahedral and binds quite tightly to the phosphate site in ASADH, but its charge density on oxygen is considerably lower than that of many tetrahedral oxyanions that do not bind to ASADH (Table 2). There is no correlation observed between the charge on the central atom and binding recognition by ASADH as either a substrate or a competitive inhibitor.

Comparative Binding Specificity. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes an oxidative phosphorylation reaction that is related to that catalyzed by ASADH. These enzymes also proceed by a common mechanism in which phosphate attack on a relatively stable acyl enzyme intermediate leads to the product. While these enzymes are mechanistically related, some differences have been found in the positioning of the active site functional groups[5] and now in the phosphate recognition site. Molybdate and phosphonate have previously been shown to be alternative substrates for GAPDH.[14,15] However, molybdate is neither a substrate nor an inhibitor for ASADH, and phosphonate is only a weak inhibitor. Vanadate is an excellent substrate for ASADH and has been demonstrated to be a substrate for GAPDH.[16] Arsenate is also an alternative substrate for both enzymes but does not show saturation behavior with GAPDH up to 80 mM,[15] in contrast to a K_m of 1.6 mM observed with ASADH. Differences are also seen in the specificity for inhibitor binding. Ions such as sulfate, nitrate, and selenate, which are weak inhibitors of GAPDH, are noninhibitory toward ASADH, and perrhenate, a good inhibitor of GAPDH, is only a very weak inhibitor of ASADH. Phosphate binding in GAPDH appears to involve hydrogen bonding to side chain serine and threonine hydroxyl groups,[17] while in ASADH the putative phosphate site contains arginines and a lysine that can provide electrostatic attraction to bind an oxyanion.[5] Electrostatic binding of oxyanions to ASADH will be enhanced by increasing the charge on the peripheral oxygens.

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