Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 63-68

AN ACID PHOSPHATASE FROM *Aspergillus ficuum* HAS HOMOLOGY TO *Penicillium chrysogenum PHOA*

Kenneth C. Ehrlich, Beverly G. Montalbano, Edward J. Mullaney, H. Charles Dischinger, Jr., and Abul H.J. Ullah

Southern Regional Research Center, USDA, New Orleans, LA 70179

Received August 23, 1994

SUMMARY: Three secreted acid phosphatases had previously been characterized from *Aspergillus ficuum* grown under conditions of limited phosphate. One of these could not be readily separated from AFPhyB, a pH 2.5 optimum acid phosphatase with phytase activity. From extensive protein sequence analysis and subsequent cloning of the gene, we have shown that the AFPhyB protein fraction contains a fourth secreted acid phosphatase (AFPhoA) that has 64% homology to a phosphate-repressible acid phosphatase from *Penicillium chrysogenum*. Garnier plot analysis revealed that the putative phosphate catalytic domain of AFPhoA at $His^{215}Asp^{216}$ is similar to those of other acid phosphatases, but that AFPhoA lacks the phosphate-binding motif RHGXRXP of known histidine phosphatases.

Aspergillus ficuum NRRL 3125 grown on cornstarch media (1) under conditions of limited phosphate, secretes high levels of several acid phosphatases (APases) (2,3,4). Because phytic acid is considered to be an antinutritional factor (for a review see 5), there has been considerable interest in using these APases as a way to decrease the amount of phytic acid in grains fed to animals. Three extracellular APases, characterized as pH optimum 5.0, 2.5 and 6.0 have previously been identified in the culture fluid (2,3,4). Two of these have activity against phytic acid, AFPhyA (pH

Abbreviations: APase(s), acid phosphatase(s); bp, base pair; AFPhyA, AFPhyB, *A.ficuum* phytase type A or type B (*myo*-inositol hexakisphosphate phosphohydrolase (EC 3.1.3.8); AFPhoA, *A. ficuum* acid phosphatase type A (EC 3.1.3.2); PCPhoA, *P. chrysogenum* acid phosphatase; EcAP, *E. coli* acid phosphatase; YstPho1, YstPho5, acid phosphatases from yeast.

0006-291X/94 $5.00

optimum 5.0) and AFPhyB (pH optimum 2.5). The protein fraction containing AFPhyB now has been shown to contain a fourth extracellular APase, AFPhoA. The structure of this protein differs significantly from the other APases and most closely resembles that of a secreted phosphate-repressible APase from *Penicillium chrysogenum* (6,7).

METHODS

Protein purification: The pH 2.5 optimum APase was purified from *A. ficuum* NRRL 3135 propagated on starch medium in shake flask cultures as previously described (3). The protein fraction, purified through a second SP-Trisacryl M chromatography step, eluted as a broad band on a SynChropak RP-8 column (0.46 x 25 cm, SynChrom, Lafayette, IN) and showed a unique N-terminal sequence upon peptide mapping of the N-terminus. This fraction was pyridylethylated as previously described (8), and 1-2 nmol was cleaved by cyanogen bromide, trypsin, chymotrypsin, clostripain, and endoproteinase Glu-C as described by Tarr (9).
Peptide sequencing: The primary structures of C18 column-purified (218TP54, Vydac, Hesperia, CA) peptides were determined using a Porton PI2090 gas phase sequencer (Beckman Instruments, Fullerton, CA). Sequence alignment and secondary structure prediction used the FASTA program of Pearson and Lipman (10) and the method of Garnier et al. (11), respectively. Protein mass analysis and pI were estimated using software from Lighthouse Data, Odense, Denmark.
Cloning and sequencing: Non-degenerate oligonucleotides (813 and 815) were synthesized on a Model 380A DNA synthesizer (Applied Biosystems, Foster City, CA), taking into account the codon bias for highly expressed genes in *Aspergillus spp.* (12). The oligonucleotides were used in a polymerase chain reaction to amplify, from *A. ficuum* DNA, an 1136-bp portion of the APase gene, which, after ^{32}P-labeling was used as a hybridization probe to screen a lambda EMBL3 *A. ficuum* DNA library. Several clones were obtained, and an *EcoRI/XhoI* fragment from one of these (lambda AF3-5) was subcloned in pBluescript (Stratagene, La Jolla, CA) to give the plasmid, pAF3-5. This plasmid was partially sequenced using Sequenase, version 2.0 (USB, Cleveland, OH) and specific synthetic oligonucleotides. The sequence of the *AFPhoA* gene from nucleotide 1402 to the poly A tail was obtained from oligo-dT primed mRNA by first-strand cDNA synthesis using a kit from Pharmacia (Piscataway, NJ). To determine the N-terminus, cDNA from randomly primed *A. ficuum* mRNA and antisense 813 (primer 813, synthesized 3′ to 5′) was directly sequenced using the fmole sequencing kit from Promega (Madison, WI).

RESULTS AND DISCUSSION

Sequence analysis of the *A. ficuum* pH 2.5-optimum APase fraction showed that it contained two different proteins. One of these, AFPhyB, has been characterized as a phytase as well as an

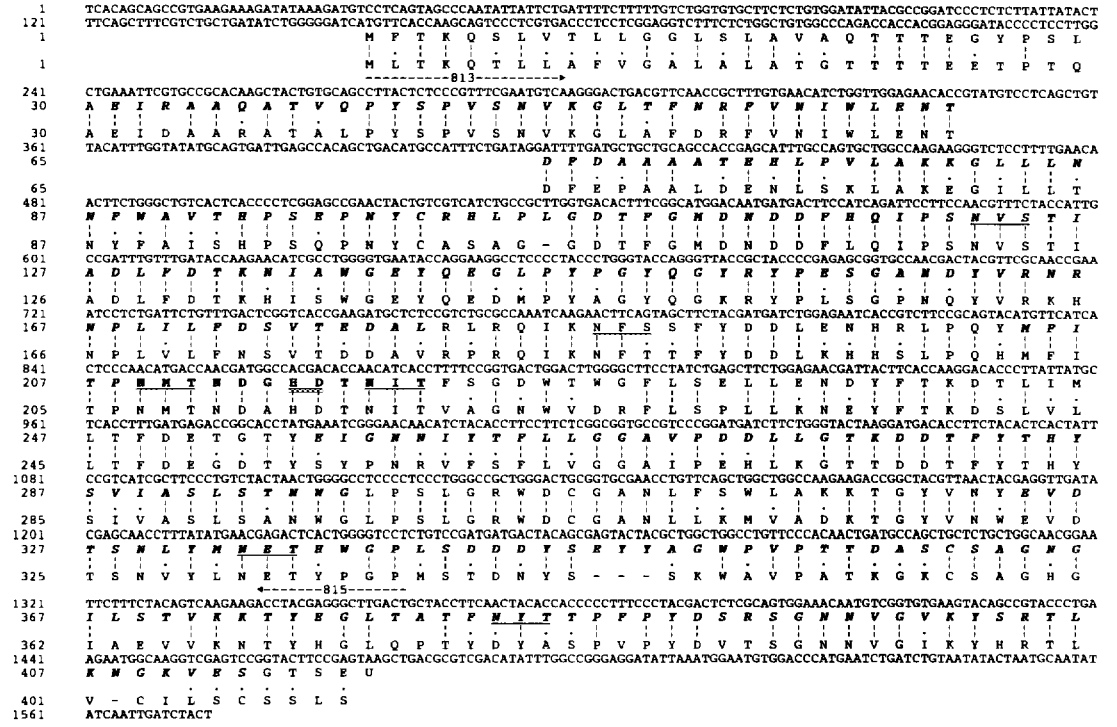

Figure 1. The nucleotide and deduced amino acid sequence of the *A. ficuum* AFPhoA gene (GenBank Accession No. L20566). The amino acid sequence is compared to *P. chrysogenum* PhoA (PIR PECAPPR)(6). Italicized and bold amino acid sequences were determined by direct amino acid sequencing as well as from translation of DNA sequence. Putative N-glycosylation sites are underlined. The location of the putative catalytic site is doubly underlined.

APase (13,14). The N-terminus of the second APase was probably blocked, since sequence analysis of the protein mixture showed only the N-terminus of AFPhyB. Extensive sequence analysis of internal peptide fragments from cyanogen bromide, trypsin, chymotrypsin, endoproteinase Glu-C, and clostripain-digested protein (Figure 1, amino acids shown in bold italics) identified a protein whose sequence showed closest homology to a previously characterized phosphate-repressible APase from *P. chrysogenum* (6,7).

Using polymerase chain reaction to generate a hybridization probe, we isolated from a genomic DNA library, a clone containing a 1500-bp DNA fragment whose deduced amino acid sequence had 64% homology to *P. chrysogenum phoA*. Like *P. chrysogenum phoA* the gene

```
AFPhoA      209 PNMTNDGHDTNIT
                ****** ******
PCPhoA      207 PNMTNDAHDTNIT
                *   *  ******
AFPhyB      330 PLFFNLAHDTNIT
                *     **  *
AFPhyA1     262 PFCDLFTHDEWIN
                      **  *
AFPhyA2     354 TLYADFSHDNGII
                     *****
EcAP        296 SVLFIAGHDTNLA
                     ***
HPAP        249 KLIMYSAHDTTVS
                     ** **
YstPho1     323 QVFFAFTHDANII
                     *** *
YstPho5     309 KVWLSFTHDTDIL
```

Figure 2. Comparison of sequences in the putative acid phosphatase catalytic site. APases shown in alignment with *A. ficuum* NRRL 3135 PhoA are *P. chrysogenum* PhoA (6), *A. ficuum* NRRL 3135 phytases (AFPhyA and AFPhyB) (13,22), *E. coli* APase (EcAP) (15), human prostate APase (HPAP) (23), and the yeast APases (YstPHO1 and YstPHO5) (24,25). Two putative sites are shown in AFPhyA. Identical amino acids are denoted by asterisks and residues that represent conservative substitutions relative to AFPhoA are underlined.

contained one 58-bp intron near its 5'-end. The location of the intron-exon boundary was confirmed by sequencing a peptide that overlapped the intron (Figure 1). The open reading frame coded for a protein with 417 amino acids with a calculated molecular weight of 46741 daltons. Six possible glycosylation sites at NX(T,S) amino acid residues are underlined in Figure 1. A possible active site for catalysis was found at His^{215} and Asp^{216}. These amino acids had previously been shown to be necessary for activity in the *E. coli* APase (EcAP). This region showed a high degree of similarity to analogous regions found in APases from a number of different species (15) (Figure 2). It should be noted that AFPhoA is very similar to AFPhyB at this site, but lacks similarity in other regions. The RHGXRXP motif, typical of many APase enzymes (15-18) was not present in AFPhoA. The N-terminal sequence of AFPhoA was compared with those of AFPhyA and AFPhyB to estimate the length of the signal peptide. For the latter two proteins the signal peptide was 19 amino acids long. Judging from this value we estimate that

the mature PhoA is 398 amino acids with a mass of 44,800 daltons for the non-glycosylated form. The estimated pI and molar extinction coefficient are 4.39 and 9.05 x 10^4, respectively.

Garnier analysis of the protein secondary structure revealed two alternating α/β domains from the N-terminus to residue 69, characteristic of phosphate-binding proteins, for example, rat APase (19) or yeast phosphoglycerate mutase (20). Analysis showed a preponderance of β-sheet domains for the rest of the protein (residues 70-412). These β-sheets may form an up-and-down antiparallel β-barrel (20). The $His^{215}Asp^{216}$ are located in a stretch of amino acids with turn and coil conformation in a hydrophilic environment as deduced by the hydropathy plot (21); this conformation is highly suitable for these residues being in the catalytic domain of the protein. Other regions of AFPhoA may play a role in substrate binding. Ostanin, et al (15) had shown, by site-directed mutagenesis, that Arg^{92} of EcAP is an essential component of the catalytic site, and most likely functions in phosphate binding. Van Etten, et al. (23) found that the region, RXRY(R,H), was conserved in many APases and appears to play a critical role in catalysis. Such positively charged regions may interact with the negatively charged phosphate during the hydrolysis reaction. AFPhoA has two RXR hydrophilic regions, the sequence, RNRN (residues 164-167) and RLRQ (residues 181-184). The former is very hydrophilic and is predicted to have a β-turn conformation favorable for placement in the catalytic domain, while the latter is somewhat less hydrophilic and is predicted to have more α-helical character. AFPhyA has an amino acid region, RQRL (residues 217-220), that may function in substrate binding of phytic acid by this phosphatase. The role of these regions in phosphatase activity awaits analysis by site-directed mutagenesis of the recombinant protein.

REFERENCES

1. Shieh, T.R. & Ware, J.H. (1968) Appl. Microbiol. **16**, 1348-13512.
2. Ullah, A.H.J. & Cummins, B.J. (1987) Prep. Biochem. **17**, 397-422.
3. Ullah, A.H.J. & Gibson, D.M. (1987) Prep. Biochem. **17**, 63-91.
4. Ullah, A.H.J. & Cummins, B.J. (1988) Prep. Biochem. **18**, 37-65.
5. Maga, J.A. (1982) J. Agric. Food Chem. **30**, 1-9.
6. Haas, H., Redl, B., Friedlin, E. & Stoeffler, G. (1992) Gene **113**, 129-133.
7. Haas, H., Redl, B., Leitner, E. & Stoffler, G. (1991) Biochim. Biophys. Acta **1074**, 392-397.
8. Thomsen, J. & Bayne, S. (1988) J. Protein Chem. **7**, 295-296.
9. Tarr, G. (1986) *In* Methods of protein microcharacterization (J.Shively, Ed.), pp. 155-194, Humana Press, Clifton, NJ.
10. Pearson, W.R. & Lipman, D.J. (1988) Proc. Natl. Acad. Sci. USA **85**, 2444-2448.
11. Garnier, J., Osguthorpe, D.J. & Robson, B. (1978) J. Mol. Biol. **120**, 97-120.
12. Lloyd, A.T. & Sharp, P.M. (1991) Mol. Gen. Genet. **230**, 288-294.
13. Ehrlich, K.C., Montalbano, B.G., Mullaney, E.J., Dischinger, H.C.,Jr. & Ullah, A.H.J. (1993) Biochem. Biophys. Res. Commun. **195**, 53-57.
14. Ullah, A.H.J. & Phillippy, B.Q. (1994) J. Agric. Food Chem. **42**, 423-425.
15. Ostanin, K., Harms, E.H., Stevis, P.E., Kuciel, R., Zhou, M.-M. & Van Etten, R.L. (1992) J. Biol. Chem. **267**, 22830-22836.
16. Ullah, A.H.J., Cummins, B.J. & Dischinger, H.C.,Jr. (1991) Biochem. Biophys. Res. Commun. **178**, 45-53.
17. Ullah, A.H.J. & Dischinger, H.C., Jr. (1993) Biochem. Biophys. Res. Commun. **192**, 747-753.
18. Ullah, A.H.J. & Dischinger, H.C., Jr. (1993) Biochem. Biophys. Res. Commun. **192**, 754-759.
19. Lindqvist, Y., Schneider, G. & Vihko, P. (1993) J. Biol. Chem. **268**, 20744-20746.
20. Richardson, J.S. (1981) *In* Advances in Protein Chemistry, Vol 34 (C.B. Anfinsen, J.T. Edsall, and F.M. Richards, Eds., pp. 167-339, Academic Press, New York, NY.
21. Kyte, J. & Doolittle, R.F. (1982) J. Mol. Biol. **157**, 105-132.
22. Van Hartingsveldt, W., Van Zeijl, C.M.J., Harteveld, G.M., Gouka, R.J., Suykerbuyk, M.E.G., Luiten, R.G.M., Van Paridon, P.A., Selton, G.C.M., Veenstra, A.E., Van Gorcom, R.F.M. & Van Den Hondel, C.A.M.J.J. (1993) Gene **127**, 87-94.
23. Van Etten, R.L., Davidson, R., Stevis, P.E., MacArthur, H. & Moore, D.L. (1991) J. Biol. Chem. **266**, 2313-2319.
24. Elliot, S., Chang, C., Schweingruber, M.E., Schaller, J., Rickli, E.E. & Carbon, J. (1986) J. Biol. Chem. **261**, 2936-2941.
25. Bajwa, W., Meyhack, B., Rudolph, H., Schweingruber, A.-M. & Hinnen, A. (1984) Nucl. Acids Res. **12**, 7721-7729.