Vol. 171, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 31, 1990 Pages 313-318

PURIFICATION AND CHARACTERIZATION OF TWO PROTEINS WITH INORGANIC PYROPHOSPHATASE ACTIVITY FROM Desulfovibrio vulgaris: RUBRERYTHRIN AND A NEW, HIGHLY ACTIVE, ENZYME

Ming-Y. Liu and Jean Le Gall

Department of Biochemistry,
School of Chemical Sciences,
University of Georgia, Athens, Georgia 30602

Received June 28, 1990

SUMMARY: The inorganic pyrophosphatase activity of a soluble extract from the strict anaerobe, sulfate-reducing, Desulfovibrio vulgaris, is readily resolved into two peaks. After purification, two active proteins with very dissimilar properties are obtained. One is the non-heme iron-containing rubrerythrin, with a specific activity of 350 pyrophosphate hydrolyzed, min^{-1}, mg $protein^{-1}$. The other, a protein of Mr = 39,000, with a specific activity of 12,000.

The first step in the dissimilatory reduction of sulfate by sulfate-reducing bacteria is the activation of this molecule by ATP-sulfurylase with the resulting formation of one molecule of adenosyl-phosphosulfate and one molecule of pyrophosphate. The accumulation of this compound is prevented by inorganic pyrophosphatases that hydrolyze it into two molecules of inorganic phosphate. Reports on the purification of this type of enzyme from Desulfovibrio vulgaris strain Hildenborough (1) and from D. desulfuricans, strain Berre-Sol (2) a nitrogen fixing organism (3), have been published. However, the enzyme has never been fully described and the specific activity of the final preparations were rather low.

Rubrerythrin, a newly discovered protein, contains both rubredoxin-like centers and a binuclear, hemerythrin-like, iron center (4), its physiological function is still unknown. A role similar to hemerythrin which is an oxygen-binding protein is very unlikely in a strict anaerobe. However, since several enzymes containing similar binuclear-iron centers, such as uteroferrin and other purple acid phosphatases have been shown to be capable of hydrolyzing pyrophosphate (5), it was logical to look for

Abbreviations used: HPLC: high-pressure liquid chromatography; EPR: electron paramagnetic resonance; EDTA: ethylene diamine tetracetate.

0006-291X/90 $1.50

such a function in the rubrerythrin isolated from D. vulgaris. We report here the results of such a study that lead to the discovery of two distinct pyrophosphatases in this organism.

MATERIALS AND METHODS

Organism and growth conditions: D. vulgaris, strain Hildenborough (NCIB 8303) was grown in the lactate-sulfate medium described by Starkey (6). The temperature was 37°C and the cells were harvested as previously described (7).

Analytical Procedures:

A Shimadzu spectrophotomer (UV265) was used for recording absolute absorption spectra. Protein homogeneity was tested with analytical polyacrylamide disc gel electrophoresis (8). Molecular weight was determined by SDS gel electrophoresis (9) and HPLC with a TSK 3000 SW molecular sieve column. Protein standards were: lysozyme (M.W. 14,400), Soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phospholipase b (92,500). Protein quantities were determined by the method developed by Lowry et al. (10). An Applied Biosystem sequencer (Model 470A) was employed according to the method of Edman and Begg (11) for N-terminal sequence. Metal content was determined by plasma emission spectroscopy, using the Jarrell-Ash Model 750 atom comp.

Amino-acid analysis were performed after hydrolysis of the samples in 6N HCl for 24, 48 and 72 hours at 110°C. For cysteine determination the samples were treated with performic acid prior to hydrolysis (14). Tryptophan was determined after treatment with thioglycolic acid (15). Samples were analyzed for individual amino acids with a Beckman HPLC equipped with a reverse phase column.

Inorganic pyrophosphatase activity was measured by the method of Thebrath, Dilling and Cypionka (12). Inorganic phosphate was estimated colorimetrically by the method of Taussky and Shorr (13). No activity could be detected if Mg^{2+} was omitted in the reaction mixtures. One unit of enzyme is the amount necessary to hydrolyze 1 μmole of pyrophosphate per min, at 25°C.

Preparation of the extract: 100 g of cells, wet weight, were mixed with an equal volume of 10 mM Tris-HCl buffer pH 7.6 (this pH was used during all further purification steps) and the resulting suspension was centrifuged at the resulting extract contained 95% of the total activity found in cells broken with a French pressure cell.

RESULTS AND DISCUSSION

As can be seen in figure 1, two peaks of activity were obtained from the very first DEAE-cellulose column after elution with a linear gradient from 10 to 200 mM Tris-HCl. The first fraction, representing 17% of the total inorganic pyrophosphatase activity, was rich in rubrerythrin and was further purified according to the method already described for this protein (4). It was found that the pyrophosphatase activity co-purified with rubrerythrin. The pure protein had a specific activity of 350 units mg^{-1} at pH 8.0.

The fraction containing 83% of the total inorganic pyrophosphatase activity was further purified by HPLC: a first step using a DEAE column (Waters 21.5 x 150 mm) lead to a fraction containing a mixture of mainly pyrophosphatase and Adenosyl-phospho-sulfate-reductase. Most of this latter protein was separated from the enzyme

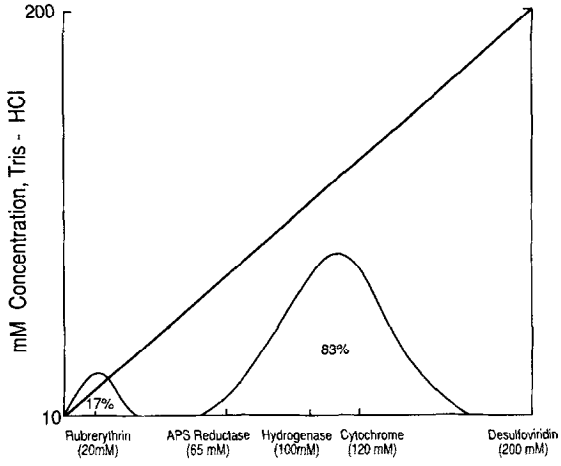

Figure 1. elution profile of the two pyrophosphatases during the first DEAE-cellulose step. Some typical Desulfovibrio proteins are indicated as markers.

by gel filtration (Beckman 3000 SW, 21.5 x 600 mm) and the same DEAE column was used in a final step that gave a fraction containing 13% of the original activity with a specific activity of 12,000, at pH 8.0 were maximum activity was found.

Properties of the high activity pyrophosphatase:

The protein was juged pure after SDS gel electrophoresis were it migrated as a single band corresponding to a molecular weight of 39,000. The enzyme is found as a monomer since a native preparation migrated with an apparent molecular weight of 36,700 in a molecular sieve column.

The only metal detected by plasma emission was zinc (one atom per mole). However, prolonged dialysis against 10 mM EDTA lead an enzyme completely

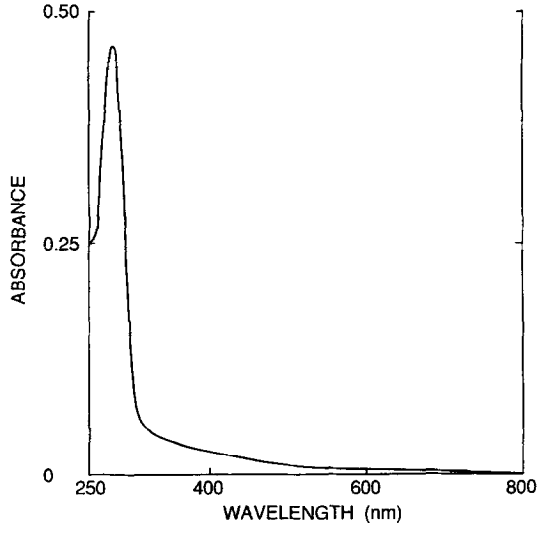

Figure 2. UV/Visible spectrum of the purified highly active pyrophosphatase.

Table I. Comparison of amino acid composition of pyrophosphatases from D. vulgaris and yeast

amino acid	D. vulgaris	yeast*
ASP	23	40
THR	58	19
SER	11	13
GLU	24	26
PRO	15	20
GLY	22	16
ALA	24	22
VAL	26	14
MET	6	2
ILE	19	27
LEU	32	17
TYR	18	11
PHE	14	11
HIS	10	6
LYS	19	28
TRP	2	6
ARG	22	6
CYS	7	1

*see ref. 16.

devoided of zinc. During this process, 40% of the activity was lost; addition of zinc chloride to the preparation did not induce any significant increase of activity. Therefore, it is not yet possible to conclude whether or not zinc is of any importance for maintaining maximum activity in this enzyme.

The optical spectrum of the enzyme is shown in Figure 2. A small absorption can be seen as a very broad shoulder centered around 400 nm. The origin of this absorption is unknown: although the protein did not contain any metal, but zinc, we checked its EPR spectra is both native and native plus dithionite forms: these spectra were absolutely flat.

The amino acid composition of the enzyme is shown in Table I. The protein is relatively rich in aromatic and histidinyl residues. The composition of the yeast inorganic pyrophosphatase is given as a comparison.

The result of the analysis of the N-terminal part of the enzyme is shown below:

```
                1                                                  10
D. vulgaris     N - [Y - T] - - - I - G - N - D - N - V - L - [T] - E - P - L - E
  yeast         T - [Y - T] - T - R - Q - I - G - A - K - N - [T] - - - - - - - - -
                1                                                  10

                                  20                                              30
D. vulgaris     S - E - I - K - T - A - G - [L] - M - [Y - K] - M - G - V - Q -
  yeast         - - - - - - - - - - - - - - [L] - E - [Y - K] - V - Y - I - E -
                                                  15
```

Although this part of the molecule has not clear homology with any other protein of known sequence, a few similarities (boxed residues) can be found with the N-

terminal part of yeast inorganic pyrophosphatase (16). It is noteworthy that the activity of this latter enzyme (7) is 20 times less than that of the D. vulgaris enzyme. This extremely high activity could be related to the fact that this protein is part of a dissimilatory pathway, i.e. sulfate respiration.

Since the specific activity of rubrerythrin was low in comparison to the other enzyme and, although the two proteins separated readily during purification, it was necessary to clearly differenciate the two activities in case that rubrerythrin should have been contaminated with some high activity enzyme. It was found that arsenate is a very potent inhibitor of the activity associated with rubrerythrin (80% inhibition at a concentration of 20 mM of arsenate) when it had very little effect on the metal-free enzyme (13% inhibition at a concentration of 66 mM of arsenate). It is interesting to note here that arsenate is a perturbant of the spectroscopic properties of uteroferrin, another binuclear iron center-containing protein (18). Preliminary results have shown that the E.P.R. spectrum of rubrerythrin is also modified upon addition of arsenate to the native enzyme (J.J.G. Moura, personal communication).

Since it has been reported that pyrophosphatase activity was reductant-activated in D. vulgaris Hildenborough extracts (4) and that the activity of rubrerythrin, having metallic, redox sensitive, centers, could well be regulated by redox conditions, a particular attention was given to this problem. The results were negative: the activity lost after addition of ferricyanide to a solution of rubrerythrin could not be recovered after addition of dithionite or ascorbate; furthermore the same reductants was not capable of increasing the activity of the as-prepared enzyme. Addition of reductants to either crude extracts of D. vulgaris or to the purified highly active enzyme also failed to increase the inorganic pyrophosphatase activity.

It remains possible that the real substrate for rubrerythrin is not pyrophosphate, but another molecule containing a pyrophosphate linkage. That rubrerythrin is a phosphatase is unlikely since the protein was totally inactive toward p-nitrophenylphosphate, a substrate commonly used in phosphatases assays (19). The highly active enzyme was also inactive toward this substrate. The presence of two pyrophosphatases in D. vulgaris has been mentioned by Baliga, Vartak and Jagannathan (20) as early as 1961: one soluble, the other insoluble. However, the extraction method used by these authors does not permit any conclusion relative to the location of those two enzymes.

The present finding of both enzymes in the soluble extract of unbroken cells is an indication of their possible periplasmic location. If this were to be confirmed it would then imply the necessity of a transport system for pyrophosphate to cross the cytoplasmic membrane, this could shed some new light on the energetics of sulfate respiration

ACKNOWLEDGMENTS

The authors thank Mr. M. Howard for his excellent technical expertise and personnel of the Fermentation Plant for growing the bacterial cells that were used in this study. Special thanks are due to Dr. J. Wunderlich for determining the N-terminal of the protein and to Dr. D. Patil for running the EPR spectrometer.

REFERENCES

1. Akagi, J.M. and Campbell, L.L. (1963) J. of Bacteriol. 86, 563-568.
2. Ware, D.A. and Postgate, J.R. (1971) J. of Gen. Microbiol. 67, 145-160.
3. Le Gall, J., Senez, J.C. and Pichinoty, F. (1959) Ann. Inst. Pasteur 96, 223-230.
4. Le Gall, J., Prickril, B.C., Moura, I., Xavier, A.V., Moura, J.J.G. and Huynh, B.-H. (1988) Biochemistry 27, 1636-1642.
5. Antanaitis, B.C. and Aisen, P. (1983) Adv. Inorg. Biochem. 5, 111-136.
6. Starkey, R.L. (1938) Arch. Microbiol. 9, 268-304.
7. Le Gall, J. and Bruschi-Heriaud, M. (1968) Structure and Function of Cytochromes. pp. 467-470, University Park Press, College Park, Maryland.
8. Brewer, J.M. and Ashworth, R.B. (1969) J. Chem. Educ. 46, 41-45.
9. Laemmli, U.K. (1970) Nature (London) 227, 680-685.
10. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
11. Edman, P. and Begg, G. (1967) Eur. J. Biochem. 1, 80-91.
12. Thebrath, B., Dilling, W. and Cypionka, H. (1989) Arch. Microbiol. 152, 296-301.
13. Taussky, H.H. and Shorr, E. (1953) J. Biol. Chem. 202, 675-685.
14. Hirs, C.H.W. (1967) Methods in Enzymol. 11, 199-203.
15. Matsubara, H. and Sasaki, R.M. (1969) Biochem. Biophys. Res. Comm. 35, 175-181.
16. Cohen, S.A., Sterner, R., Keim, P.A. and Heinrikson, R.L. (1978) J. Biol. Chem. 253, 889-897.
17. Ridlington, J.W., Yang, Y. and Butler, L.G. (1972) Arch. Biochem. Biophys. 153, 714-725.
18. Doi, K., Antanaitis, B.C. and Aisen, P. (1988) Structure and Bonding 70, pp 1-26, Springer-Verlag Berlin Heidelberg.
19. Snyder, S.L., Wilson, I. and Bauer, W. (1972) Biochim. Biophys. Acta 258, 178-187.
20. Baliga, B.S., Vartak, H.G. Jagannathan, V. (1961) J. of Scientific and Indust. Res. 20C, 33-40.