Vol. 100, No. 4, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 30, 1981 Pages 1555-1561

ISOLATION OF AN ACID PROTEASE FROM RABBIT RETICULOCYTES AND EVIDENCE FOR ITS ROLE IN PROCESSING REDOX PROTEINS DURING ERYTHROID MATURATION

Dorothy A. Schafer and Donald E. Hultquist

Department of Biological Chemistry
The University of Michigan
Ann Arbor, Michigan 48109

Received April 27,1981

Summary: A protease which generates a soluble hemepeptide from bovine liver microsomal cytochrome b_5 has been isolated from the membrane fraction of rabbit reticulocytes. Inhibition by pepstatin and an acidic pH optimum indicate that the protease belongs to the acid protease class. Little cytochrome b_5-processing activity is observed in rabbit erythrocytes. We suggest that the protease may be involved in the processing which generates the proteins of the methemoglobin reduction system from their membrane-bound precursors during the maturation of the erythroid cell.

The methemoglobin reduction system of erythrocytes is composed of cytochrome b_5 reductase and cytochrome b_5 (1). In contrast to the microsomal cytochrome b_5/cytochrome b_5 reductase system of the liver, the red cell redox proteins are soluble enzymes devoid of any membrane-binding capability. Amino acid analysis (2) and tryptic peptide mapping (3) have established that the two forms of soluble cytochrome b_5 of bovine erythrocytes have amino acid sequences which correspond to residues 1-97 and 1-95 of the hydrophilic domain of the liver microsomal protein. Likewise, immunological studies (4) and protease modification studies (5) of cytochrome b_5 reductase from erythrocytes and from endoplasmic reticulum have indicated a similar identity between the hydrophilic domains of the two flavoproteins.

The recognition of the similarities between the catalytic domains of the microsomal proteins and the soluble red cell proteins has led to the postulation that the soluble red cell proteins are derived from the

Abbreviations used: ACD, acid-citrate-dextrose; PBS, phosphate-buffered saline containing 0.01 M potassium phosphate, 0.14 M sodium chloride; PMSF, phenylmethylsulfonylfluoride; TPCK, L-1-tosylamide-2-phenylethyl-chloromethylketone; TLCK, N-α-p-tosyl-L-lysine chloromethylketone.

0006-291X/81/121555-07$01.00/0

membrane-bound forms via post-translational proteolytic processing during the maturation of bone marrow erythroid cells to circulating erythrocytes (2). Studies of the redox proteins of the Friend erythroleukemia cell, a prototype of an immature erythroid cell, have revealed the presence of only the membrane-bound forms of cytochrome b_5 and cytochrome b_5 reductase, lending additional support for a precursor-product relationship between the membrane-bound proteins of the immature cell and the soluble proteins of the mature erythrocytes (6).

We have observed (3) the ability of a bovine liver lysosomal fraction to degrade microsomal cytochrome b_5 to a soluble form identical to one of the forms of erythrocyte cytochrome b_5, implicating lysosomal proteases as the processing enzymes which degrade the microsomal proteins in erythroid cells. We now report the detection of a proteolytic activity in rabbit reticulocytes which will degrade both microsomal cytochrome b_5 and reductase to soluble proteins. This protease could be a component of the processing system of erythroid cells which generates the cytoplasmic methemoglobin reduction system during erythroid cell maturation.

EXPERIMENTAL

Materials. Detergent-extracted microsomal cytochrome b_5 from bovine liver was prepared according to the method of Ozols (7). Bovine erythrocyte cytochrome b_5 was purified by a modification of the procedure of Douglas and Hultquist (2).

Sigmacell and Percoll were purchased from Sigma. Fibrous cellulose powder (CF-11) was purchased from Whatman. All chemicals were reagent grade.

Preparation of erythrocytes. Blood was drawn from the ear artery of a male, New Zealand rabbit using ACD as anticoagulant. Erythrocytes were purified from the whole blood by a combination filtration-density gradient sedimentation technique. Filtration through cellulose was carried out by a modification of the method described by Beutler et al. (8). The whole blood was centrifuged 10 min at 500 x g. The plasma was removed and retained and the "buffy coat" cells removed by aspiration. The plasma was then added back to the red cells to give a hematocrit of 50. Five-ml aliquots of the red cell suspension were allowed to filter through 1 x 2 cm columns of cellulose-Sigmacell (1:1 w/w) equilibrated in PBS. The filtration step was repeated and the cells were collected by centrifugation at 600 x g for 10 min. Again, the cells were suspended in PBS to a hematocrit of 50.

Density gradients were generated by centrifugation of 12 ml of Percoll (starting density = 1.09 g/ml) in 15 ml Corex test tubes for 30 min at 22,000 x g. One ml of the red cell suspension was applied to the top of each gradient and the cells were sedimented by centrifugation at 800 x g for 15 min in a

swinging bucket rotor. Erythrocytes, which pelleted near the bottom of this gradient, were removed and washed 3 times with PBS to remove residual Percoll.

Preparation of reticulocytes. Reticulocytes were obtained from rabbits made anemic by daily bleeding. Purification of reticulocytes was identical to the procedure described for erythrocytes. Red blood cells and white blood cells were counted using a Coulter Counter.

Membrane preparation. Membranes from reticulocytes and erythrocytes were prepared as described by Hanahan and Ekholm (9). An aliquot of the red blood cell suspension which contained 2 x 10^9 cells was added to 12 ml of cold 0.005 M potassium phosphate, pH 8.0. Membranes were obtained by centrifugation at 22,000 x g for 20 min and the membrane fraction was washed three times with the same buffer. Membranes were stored at -20°C.

Soluble protease preparation. An aliquot of the membrane preparation was mixed vigorously on a vortex mixer with an equal volume of 0.2 M sodium maleate, pH 5.75. The supernatant fraction obtained after centrifugation at 22,000 x g for 15 min was used as the protease preparation.

Proteolytic assay. Proteolysis of cytochrome b_5 was assayed using detergent-extracted bovine liver microsomal cytochrome b_5 as substrate. Incubation of cytochrome b_5 (1 nmol) and protease was carried out at 37°C in 0.1 M sodium maleate, pH 5.75, containing 0.3% (v/v) Triton X-100. Proteolysis was quantitated using PAGE in 11% or 15% polyacrylamide gels in the absence of detergents (10). In this gel system, the microsomal cytochrome barely penetrates into the gel, whereas any "solubilized" species without a hydrophobic tail will rapidly migrate. The solubilized cytochrome was quantitated by photometric scanning of the gel at 413 nm. The area under the peaks in the photometric trace of the gel was calculated as a measure of proteolysis. This process was facilitated by computerized data acquisition, storage, and manipulation.

RESULTS and DISCUSSION

The reticulocyte membrane protease converts a preparation of detergent-extracted bovine liver microsomal cytochrome b_5 to a smaller heme-containing peptide. Fig. 1 shows the time course for the proteolysis of microsomal cytochrome b_5 by the protease extracted from reticulocyte-enriched blood cell membranes and from erythrocyte membranes. The membranes derived from reticulocyte-enriched blood cells possess 6-7 times the amount of protease activity detected in erythrocyte membranes. In addition, the amount of protease activity is independent of the number of leucocytes present in the blood cell preparations.

The hemepeptide derived from bovine microsomal cytochrome b_5 migrates as a single form with an R_f value of 0.49 in 15% polyacrylamide gels. In contrast, the two forms of bovine erythrocyte cytochrome b_5 migrate faster in this

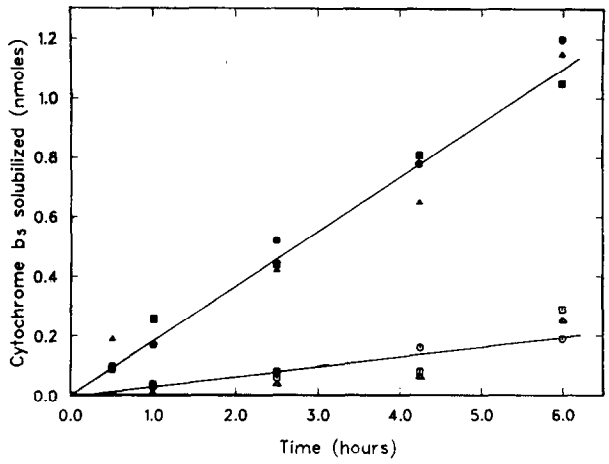

Fig. 1. Solubilization of microsomal cytochrome b_5 by protease extracted from membranes of erythrocyte and reticulocyte-enriched blood cells. One nmole of bovine liver microsomal cytochrome b_5 was incubated at 37°C with protease in 0.1 M sodium maleate, pH 5.75, 0.3% (v/v) Triton X-100. The protease fraction was prepared from blood cells containing varying amounts of leucocytes. Proteolysis was quantitated as described in Experimental. ■, reticulocyte-enriched membranes containing 0.32% leucocytes; ●, reticulocyte-enriched membranes containing 0.072% leucocytes; ▲, reticylocyte-enriched membranes containing 0.034% leucocytes; □, erythrocyte membranes containing 0.11% leucocytes; ○, erythrocyte membranes containing 0.04% leucocytes; △, erythrocyte membranes containing 0.015% leucocytes.

electrophoresis system with R_f values of 0.59 (residues 1-97) and 0.63 (residues 1-95). Consideration of the electrophoretic migration of erythrocyte cytochrome b_5 and of the primary structure of microsomal b_5 allows us to conclude that the hemepeptide obtained after digestion by the reticulocyte membrane protease is larger than the forms of erythrocyte b_5 and that cleavage is probably occurring in a region of the hydrophobic tail.

Bovine microsomal cytochrome b_5 reductase and rabbit liver microsomal cytochrome b_5 are also substrates for the reticulocyte protease when assayed at conditions used for proteolysis of bovine microsomal cytochrome b_5. Each of these substrates is also converted to a smaller form which lacks the hydrophobic tail.

The pH dependence for the digestion of microsomal cytochrome b_5 is shown in Fig. 2. Optimal activity is observed at pH 5.75; however, it is possible that proteolysis may be greater at lower pH values. Denaturation of the cytochrome in acidic solution prevents reliable assay below pH 5.5.

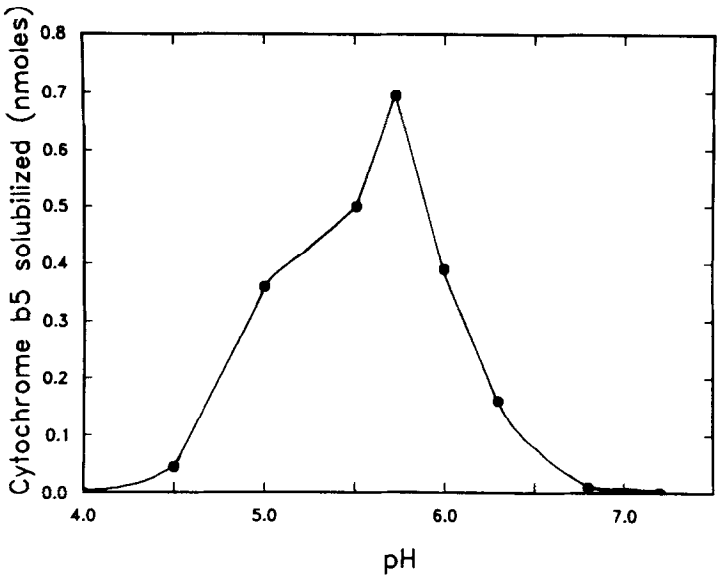

Fig. 2. Effect of pH on the proteolysis of microsomal cytochrome b_5 by reticulocyte protease. One nmole of bovine microsomal cytochrome b_5 was incubated for 1 h at 37°C with 25 µl of protease preparation. pH was adjusted by dialysis against the following buffers: 0.1 M sodium acetate, pH 4.0-5.5; 0.1 M sodium maleate, pH 5.7-6.3; 0.1 M sodium phosphate, pH 6.8-7.3. All assays contained 0.3% (v/v) Triton X-100. The protease fraction was prepared from membranes and proteolysis was quantitated as described in Experimental.

Consequently, all assays have been carried out at pH 5.75, providing for optimal proteolysis.

Pepstatin is the only protease inhibitor tested which completely prevents the processing of microsomal cytochrome b_5 to a "soluble" form. Table I shows the effects of a number of protease inhibitors on the b_5-processing activity. In addition, PMSF, TLCK, or EDTA do not inhibit the protease. Inhibition by low concentrations of pepstatin and the relatively low pH requirement strongly suggest that this protease belongs to the acid protease group.

Triton X-100 is strictly required for proteolysis of microsomal cytochrome b_5; the optimum concentration of Triton X-100 is 0.3% (v/v). The detergent may have a direct effect on the protease itself, or may act simply to release the "monomer" form of microsomal cytochrome b_5 from the large aggregate which is known to exist when b_5 is in the absence of detergents.

We report that the protease is easily liberated from the reticulocyte

TABLE I. Effects of Protease Inhibitors

Inhibitor	Concentration (mM)	% activity
None		100
Methanol	1300	95
Pepstatin	0.011	0
TPCK	1.60	83
Hemin	0.17	108
Leupeptin	6.40	109
N-ethylmaleimide	1.60	102
Cysteine	1.60	108

Inhibitors were incubated with protease preparation for 15 min at 37°C before the addition of microsomal cytochrome b_5. TPCK, pepstatin and hemin were added as methanol solutions; the final methanol concentration was 1300 mM.

membrane fraction by washing the membranes in 0.1 M sodium maleate, pH 5.75, suggesting a weak association of the protease with the membrane. We have not eliminated the possibility that the protease is actually a cytosolic enzyme which becomes associated with the membrane under conditions of hypotonic cell lysis. Cytoplasmic acid protease activities have been reported to be present in red cell lysates which have been obtained by freeze-thawing (11).

Membrane-associated protease activity in erythrocytes and reticulocytes has been reported previously (12, 13, 14, 15). Pontremoli *et al.* described three acidic proteases from human erythrocyte membranes which vary in their pH optima, molecular weight and sensitivity to pepstatin (14). Higher levels of these membrane-associated proteases were reported to be present in reticulocytes than in erythrocytes. It is possible that the cytochrome b_5-processing enzyme we find in reticulocytes is similar to one of these previously described acidic protease activities of erythrocytes.

Proteases are likely to be involved in the dramatic reconstruction of the immature erythroid cell as it becomes a circulating erythrocyte. We propose that the reticulocyte protease described here may be important in the proteolytic processing of the proteins of the cytochrome b_5 reduction system during erythroid cell maturation. This processing results not only in formation of cytoplasmic cytochrome b_5 and b_5 reductase, but also in a change in function of the electron transport system to one which reduces methemoglobin

in the mature red cell. Further studies of the reticulocyte protease may allow us to consider whether a defect in the proteolytic processing of the membrane-bound cytochrome b_5 reduction system can account for those cases of methemoglobinemia (16) in which methemoglobin reduction functions inefficiently in erythrocytes while the fatty acid desaturation pathway functions normally in other tissues.

ACKNOWLEDGEMENTS

We are especially grateful to Dr. David A. Juckett for the design and implemention the gel scanner-computer anaylsis system. This work was supported by U.S. Public Health Service Research Grant AM-09250 and Training Grant GM-00187 and by an American Cancer Society Institutional Grant.

REFERENCES

1. Hultquist, D.E. and Passon, P.G. (1971) Nature New Biol. 229, 252-254
2. Douglas, R.H. and Hultquist, D.E. (1978) Proc. Natl. Acad. Sci. USA. 75, 3118-3122
3. Hultquist, D.E., Slaughter, S.R., Douglas, R.H., Sannes, L.J. and Sahagian, G.G. (1978) In Brewer, G.J. (ed.): "Proc. 4th Intl. Conf. Red Cell Metabolism and Function," New York: Alan R. Liss, 199-211
4. Leroux, A., Torlinski, L. and Kaplan, J-C. (1977) Biochem. Biophys. Acta 481, 50-62
5. Hultquist, D.E., Peters, C.L. and Schafer, D.A. (1979) Proc. XIth Intl. Cong. Biochem. 490:Abs.
6. Slaughter, S.R. and Hultquist, D.E. (1979) J. Cell Biol. 83, 231-239
7. Ozols, J. (1974) Biochemistry 13, 426-434
8. Beutler, E., West, C. and Blume, K.G. (1976) J. Lab. Clin. Med. 88, 328-333
9. Hanahan, D.J. and Ekholm, J.E. (1974) Meth. Enz. 31, 168-172
10. Brewer, J.M. and Ashworth, R.B. (1969) J. Chem. Educ. 46, 41-45
11. Pontremoli, S., Melloni, E., Salamino, F., Sparatore, B., Michetti, M., Benatti, U., Morelli, A. and DeFlora, A. (1980) Eur. J. Biochem. 110, 421-430
12. Reichelt, D., Jacobsohn, E. and Haschen, R.J. (1974) Biochem. Biophys. Acta 341, 15-26
13. Murakami, T., Suzuki, Y. and Murachi, T. (1979) Eur. J. Biochem. 96, 221-227
14. Pontremoli, S., Salamino, F., Sparatore, B., Melloni, E., Morelli, A., Benatti, U. and DeFlora, A. (1979) Biochem. J. 181, 559-568
15. Pontremoli, S., Sparatore, B., Melloni, E., Salamino, F., Michetti, M., Morelli, A., Benatti, U. and DeFlora, A. (1980) Biochem. Biophys. Acta 630, 313-322
16. Kaplan, J-C., Leroux, A. and Beauvais, P. (1979) C.R. Soc. Biol. 173, 368-379