ACTA CHEMICA SCANDINAVICA 25 (1971) 1457–1460

Membrane-bound Inorganic Pyrophosphatase of Human Erythrocytes

CHRISTINA GEZELIUS-KJÄLLMAN, GUNNAR RONQUIST and GUNNAR ÅGREN

Institute of Medical Chemistry, University of Uppsala, S-751 22 Uppsala 1, Sweden

Inorganic pyrophosphatase has been shown to occur in a small but reproducible quantity in the membraneous part of human erythrocytes, using a radiometric assay procedure.

The specific activity of the soluble part (hemolysate) calculated as enzyme activity per mg protein was 2.6 times that of the ghosts, which represents relatively intact membrane structures. The ghosts, on the other hand, showed a specific activity over tenfold that of a membrane fraction, which represents disintegrated membrane structures.

Evidence is given that not all of the pyrophosphate molecules in the assay medium are accessible to the inorganic pyrophosphatase of the ghosts, indicating an interior localization of some of the enzyme molecules of the ghosts.

Unlabelled ATP in the assay medium reduces the activity of the inorganic pyrophosphatase of the ghosts. Possible explanations of the inhibition are discussed.

Since the work of Roche,[1] it is well known that inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1.) occurs in the interior of mammalian erythrocytes.[2–6] It has been claimed that this enzyme does not exist at all in the membraneous part of the erythrocyte but only in a soluble form of the hemolysate.[2,4] However, particulate pyrophosphatases of broad specificity have been reported to occur in the skin, liver and kidney of the rat.[7]

Furthermore, recent work has shown that several erythrocyte enzymes are associated with the membraneous structure to a varying degree (for a review, see Ronquist [8]). For that reason it was of interest to see if the presence of the inorganic pyrophosphatase of human erythrocytes could be established to any extent in the membraneous structure, when using a sensitive radiometric assay method. The present work gives strong evidence that a small but reproducible part of the inorganic pyrophosphatase activity of the human erythrocyte is associated with the membraneous structure.

MATERIAL AND METHODS

Analytical methods. Nitrogen was determined by a micro-Kjeldahl method, using a nitrogen to protein conversion factor of 6.25.[9]

The procedure for the radioactive measurements has been described previously.[10]

Special chemicals. (^{32}P)tetrasodiumpyrophosphate was purchased from the Radiochemical Centre, Amersham, Bucks, England, with a specific activity of 70.5 mCi/mmol. It was used without further purification.

Unlabelled tetrasodiumpyrophosphate was obtained from E. Merck AG, Darmstadt, West Germany. ATP (sodium salt) and glutathione (reduced form) was obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Preparation of hemolysate, ghosts and membrane fraction. Venous blood samples from healthy blood donors were collected in heparinized tubes at the Blood Transfusion Centre, University Hospital, Uppsala (by courtesy of Dr. C. F. Högman). The blood was centrifuged at 650 *g* for 20 min, and the plasma and the buffy coat layer were removed. The red cells were washed 3 times with 3 vol. of 0.155 M sodium chloride.

The further preparation of hemolysate, ghosts and membrane fraction has been described in detail elsewhere.[10] All preparative steps were carried out at 4°C.

Assay of pyrophosphatase activity. Enzymatic hydrolysis of (^{32}P)tetrasodiumpyrophosphate was assayed by a radiometric determination of liberated (^{32}P)orthophosphate after isolating the latter from the labelled pyrophosphate, using a two-phase separation system.[12]

The reaction mixture (3.0 ml) contained 50 μmol of tris-acetic acid buffer, pH 7.5, 200 μmol of NaCl, 25 μmol of KCl, 2.5 μmol of glutathione (reduced form),[11] 6.8 μmol of (^{32}P)tetrasodiumpyrophosphate, 13.6 μmol of $MgCl_2$,[3] and an appropriate amount of membraneous material or hemolysate. Moreover, in some experiments the reaction mixture also contained unlabelled ATP in varying concentrations (2.0 – 14.0 μmol). In these cases, $MgCl_2$ was added in an additional amount, corresponding stoichiometrically to the amount of unlabelled ATP added.

The reaction was initiated by the addition of membraneous material or hemolysate, incubated at 37°C for 60 min, and terminated by transferring 0.5 ml of the reaction mixture into a solution containing silicotungstate and isobutylalcohol-benzene, as described by Martin and Doty. [12] A control without membraneous material as well as hemolysate was always run, to correct for non-enzymatic breakdown of (^{32}P)pyrophosphate. Enzyme activity is expressed as μmol of (^{32}P)orthophosphate liberated per mg of protein (membraneous material or hemolysate), if not otherwise stated. Each assay was performed in duplicate.

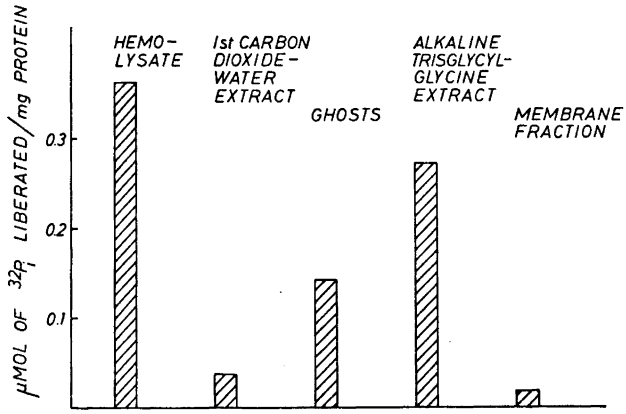

Fig. 1. Specific activity of inorganic pyrophosphatase of various erythrocyte fractions. For further details, see text.

RESULTS AND DISCUSSION

Fig. 1 illustrates the specific activity of inorganic pyrophosphatase of various fractions of human erythrocytes. The highest activity is found in the hemolysate, and was 2.6 times that of the ghosts.

However, the specific activity of the ghosts, which represent relatively intact membraneous structures,[13] was 10.8 times that of the membrane fraction, which is disintegrated and of morphologically pure membraneous origin.[13] The disintegration procedure is achieved by treating the ghosts with an alkaline tris-glycylglycine buffer,[10] and accordingly this buffer extract had a relatively high specific activity (Fig. 1). Therefore, most of the membraneous-bound pyrophosphatase activity was eluted during the disintegration procedure. The alkaline tris-glycylglycine extract of human erythrocyte ghosts has been extensively studied in our laboratory, and found to incorporate (^{32}P)orthophosphate into various phosphopeptides as well as into phosphorylethanolamine and phosphorylcholine.[14]

The total inorganic pyrophosphatase activity of various fractions from 100 ml of erythrocytes is shown in Table 1. It is seen that the predominating

Table 1. Total inorganic pyrophosphatase activity of various fractions from 100 ml of human erythrocytes. Enzyme activity expressed as μmol (^{32}P)orthophosphate liberated/h at 37°C.

Hemolysate	11481
1st Carbon dioxide-water extract	202.0
Ghosts	15.7
Alkaline tris-glycyl-glycine extract	42.6
Membrane fraction	1.4

part of the inorganic pyrophosphatase occurs in the hemolysate. The ghosts contain 0.14 % of the activity of the hemolysate and show 11 times more pyrophosphatase activity than the membrane fraction. An interesting point was the constant finding that the total activity of the membrane fraction and the alkaline tris-glycylglycine extract greatly exceeded that of the ghosts. The conclusion might be that only a minor part of the inorganic pyrophosphatase of the ghosts could have reacted with its substrate used in the assay. Therefore, some of the enzyme molecules might have been located in the interior of the ghost membranes and would not be accessible to the (^{32}P)-inorganic pyrophosphate.

Fig. 2 illustrates the reciprocal relationship between the addition of increasing amounts of unlabelled ATP to the assay medium and the decreasing liberation of (^{32}P)orthophosphate from the labelled inorganic pyrophosphate. This interesting relationship has not so far been further investigated. One explanation may be a broad specificity of the membraneous-bound enzyme which has been observed for particulate pyrophosphatases of, *e.g.*, rat liver,[7] contrary to the very strict specificity of the (soluble) inorganic pyrophosphatase from the hemolysate of human erythrocytes.[6] The unlabelled ATP could

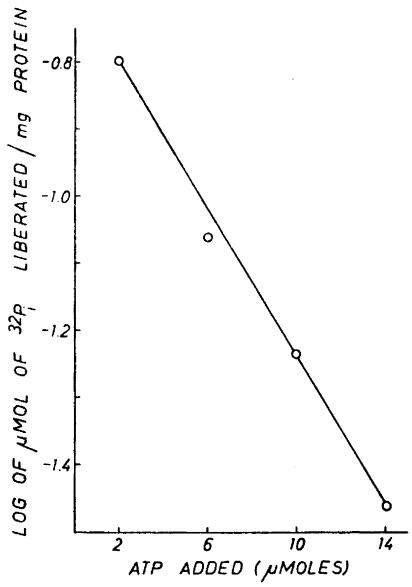

Fig. 2. Inhibiting effect of the inorganic pyrophosphatase of ghosts by varying the concentration of unlabelled ATP in assay medium. For further details, see text.

therefore interact with the labelled inorganic pyrophosphate at the centre of the membraneous-bound pyrophosphatase. It might also be possible that the ATP added to the assay medium could induce a conformational change,[15,16] and consequently provide other mechanisms for the reaction of the membraneous enzyme with its substrate.

Acknowledgement. This investigation was supported by a grant from the *Swedish Medical Research Council* (project No. B69-13X-228-05A).

REFERENCES

1. Roche, J. *Biochem. J.* **25** (1931) 1724.
2. Clarkson, E. M. and Maizels, M. *J. Physiol.* **116** (1952) 112.
3. Bloch-Frankenthal, L. *Biochem. J.* **57** (1954) 87.
4. Malkin, A. and Denstedt, O. F. *Can. J. Biochem. Physiol.* **34** (1956) 121.
5. Scheuch, D. and Rapoport, S. *Acta Biol. Med. Ger.* **7** (1961) 113.
6. Pynes, G. D. and Younathan, E. S. *J. Biol. Chem.* **242** (1967) 2119.
7. Schliselfeld, L. H., van Eys, J. and Touster, O. *J. Biol. Chem.* **240** (1965) 811.
8. Ronquist, G. *Acta Universitatis Upsaliensis, Abstracts of Uppsala Dissertations in Medicine*, No. 54 (1968).
9. Munro, H. N. In Munro, H. N. and Allison, J. B. *Mammalian Protein Metabolism*, Academic, New York and London 1964, Vol. II.
10. Ronquist, G. *Acta Chem. Scand.* **21** (1967) 1484.
11. Rapoport, S. and Scheuch, D. *Nature* **186** (1960) 967.
12. Martin, J. B. and Doty, D. M. *Anal. Chem.* **21** (1949) 965.
13. Nilsson, O. and Ronquist, G. *Biochim. Biophys. Acta* **183** (1969) 1.
14. Ågren, G. and Ronquist, G. *Acta Chem. Scand.* **18** (1964) 2029.
15. Kavanau, J. L. *Structure and Function in Biological Membranes*, Holden-Day, San Francisco 1965, Vol. I, p. 66.
16. Kavanau, J. L. *Structure and Function in Biological Membranes*, Holden-Day, San Francisco 1965, Vol. II, p. 326.

Received September 5, 1970.