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## Glycogen-storage diseage Type VIa: Low phosphorylase kinase activity caused by a low enzyme–substrate affinity

Three years ago we reported[1] that in some patients with glycogen-storage disease Type VI, there was a greatly diminished activity of phosphorylase kinase (ATP:phosphorylase phosphotransferase, EC 2.7.1.38) in the leucocytes. We suggested that the low kinase activity was responsible for the low phosphorylase ($\alpha$-1,4-glucan:orthophosphate glucosyltransferase, EC 2.4.1.1) activity in white blood cells when assayed in the absence of AMP. Since then we have shown that this type of glycogen disease shows X-chromosomal inheritance[2] and we proposed to designate this disease as Type VIa glycogen-storage disease in order to differentiate it from a second, very similar disease, Type VIb, in which leucocyte (and liver) phosphorylase activity is also low but phosphorylase kinase activity is normal[3]. We now show that the low phosphorylase kinase activity is due to altered kinetic properties of the enzyme.

Leucocytes were isolated by the technique previously described (see *e.g.* ref. 4) or by dextran sedimentation, mixing 10 ml of freshly drawn heparin blood with 20 ml cold dextran solution (30 mg/ml dextran, mol. wt. 200 000–275 000, in 0.15 M NaF). After sedimentation in ice for 20 min, the upper layer was pipetted off, diluted with 2 vol. of cold water in order to hemolyse residual red cells and immediately centrifuged for 5 min at 3000 rev./min in a clinical centrifuge. The pellet was washed twice in 0.05 M NaF.

All values shown in this article were obtained by enzyme assays in leucocytes which were isolated elsewhere by one of the two methods indicated. After isolation the cells were covered with a small volume of 0.05 M NaF and frozen in dry ice. The cells were shipped by air to our laboratory in dry ice. After arrival the tubes were thawed and the cells were homogenized in 0.05 M NaF as described before[4]. Protein and phosphorylase were determined as described before[4].

Phosphorylase kinase activity was determined in principle as described by KREBS *et al.*[5], following the activation of purified, 3 times crystallized rabbit muscle phosphorylase *b*[6] by the leucocyte extract. Good agreement was obtained when kinetic parameters were determined in leucocyte extracts obtained from one blood sample by either of the two methods described above.

Storage of frozen cells or homogenates, even at the temperature of drye ice, causes a gradual increase of $K_m$ for phosphorylase. Freshly isolated leucocytes, of normal subjects homogenized and assayed immediately after isolation show $K_m$ values for phosphorylase from 10–70 units of phosphorylase per ml reaction mixture. As can be seen in Table I, control values in samples that have been frozen are generally higher. In view of the different times involved in shipments of the samples it is best to compare values within the same experiment only. It is clear from Table I that the lowest $K_m$ value in leucocytes of a patient with Type VIa glycogen disease is at least 8 times higher than a comparable control value. On the average, patients show a 20-fold higher $K_m$ than their controls. Heterozygotes show intermediate $K_m$ values.

Maximal velocities are in the same range for patients, heterozygotes and controls. If anything patients show a somewhat higher $v_{max}$ than the other two groups. Phosphorylase kinase in leucocyte extracts of patients and heterozygotes shows the

TABLE I

KINETIC PROPERTIES OF PHOSPHORYLASE KINASE IN HUMAN LEUCOCYTES

Leucocytes were isolated as described in the text, frozen in dry ice and shipped by air at dry ice temperature.

Phosphorylase kinase was assayed by adding 20 $\mu$l of a 14 000 $\times$ $g$ supernatant of a leucocyte homogenate (10–30 $\mu$g of protein) to 100 $\mu$l of a reaction mixture containing 50mM Tris–$\beta$-glycerophosphate buffer (pH 6.8), 3.6 mM ATP (pH 6.8), 12 mM magnesium acetate and varying concentrations of phosphorylase $b$, namely 40–260 units*/ml. The digest was incubated for 30 min at 30°. The kinase reaction was then stopped by addition of 2.0 ml 40 mM glycerophosphate, 30 mM cysteine-HCl, 5 mM EDTA (pH 6.8). In a control tube the glycerophosphate–cysteine–EDTA mixture was added before the leucocyte protein. Phosphorylase $a$, formed by the kinase action, was then determined in duplicate in 50 $\mu$l samples by adding these to 50 $\mu$l of an assay mixture containing 150 mM glucose 1-phosphate (pH 6.8), 20 mg/ml AMP-free glycogen and incubating for 15 min at 30°. The reaction was stopped by the addition of 1.0 ml of 0.036 M $H_2SO_4$. Phosphate in this mixture was determined with ammonium molybdate, using $FeSO_4$ as a reducing agent. The phosphorylase $a$ content was corrected for phosphorylase $a$ in the control tube. $K_m$ and $v_{max}$ were determined by plotting $1/v$ against $1/S$. The $K_m$ for phosphorylase $b$ is expressed as units of phosphorylase $b$ per ml reaction mixture during the actual kinase assay. The $v_{max}$ is expressed as units of phosphorylase activated per min per mg of leucocyte protein in a 14000 $\times$ $g$ supernatant.

| *Experiment* | *Subject* | *Sex* | *$K_m$ for phosphorylase (units/ml)* | *$v_{max}$ (units/min per mg protein)* |
|---|---|---|---|---|
| *Normal subjects* | | | | |
| 1 | N.B. | Male | 62 | 1.6 |
| 2 | F.O | Male | 135 | 2.0 |
| 3 | P.H. | Male | 130 | 1.5 |
| | C.R. | Female | 185 | 1.0 |
| 4 | C.U. | Male | 62 | 0.9 |
| | A.Bn. | Female | 50 | 1.1 |
| *Patients with glycogen-storage disease Type VIa*** | | | | |
| 1 | L.B. | Male | 2000 | 3.0 |
| 2 | T.O | Male | 1667 | 3.0 |
| 3 | H.Ke | Male | 1429 | 2.5 |
| | G.K. | Male | 5000 | 2.4 |
| 4 | H.H. | Male | 1000 | 2.5 |
| | A.Bn. | Male | 430 | 1.5 |
| *Heterozygotes* | | | | |
| 2 | M.O. | Female | 500 | 1.7 |
| 3 | A.Ke. | Female | 830 | 1.7 |
| | I.K. | Female | 500 | 1.9 |
| 4 | C.Bn. | Female | 182 | 2.0 |
| | M.H. | Female | 167 | 0.8 |
| 5 | F.M. | Female | 480 | 1.2 |

* A unit is the amount of phosphorylase that converts 1 $\mu$mole of substrate in 1 min at 30°.

** As ascertained by the following two criteria: (1) low leucocyte phosphorylase activity in the absence of AMP; (2) low leucocyte phosphorylase kinase activity when assayed under conditions described before[1,4].

same $K_m$ for $MgATP^{2-}$ as controls, namely 0.07–0.2 mM. A high $K_m$ for phosphorylase is also observed in erythrocytes of patients with Type VIa glycogen disease.

It has been shown by KREBS *et al.*[5] that phosphorylase kinase exists in two forms in rabbit skeletal muscle, an inactive form which has a very high $K_m$ for phosphorylase at neutral pH and an active form which has a lower $K_m$. The inactive form can be activated by a cyclic AMP-dependent protein kinase[7]. This protein kinase will also

phosphorylate proteins like casein and histone[7,8]. Preliminary experiments indicate that the rate of, cyclic AMP-dependent, histone phosphorylation is equal in leucocytes of controls and patients. If this can be confirmed for the phosphorylation (and activation) of non-activated phosphorylase kinase this would mean that the defect in the patients is either a defective phosphorylase kinase or excessively rapid inactivation of the kinase.

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