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ACQUIRED PYRUVATE KINASE DEFICIENCY.

THE EFFECT OF MALEIC ACID UPON HUMAN ERYTHROCYTE PYRUVATE KINASE

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Summary

1. Maleic acid is shown to be able to bind the thiol compound 2-mercaptoethanol. This is fully consistent with the data of Morgan and Friedman (1938).

2. Human erythrocyte pyruvate kinase dissolved and quantitated in Tris-maleate shows a loss of positive homotropic interactions, as compared to the same preparation in Tris-HCl. Hill coefficients (n) of $n = 1.0—1.2$ and $n = 1.6—1.8$ are obtained in Tris-maleate and Tris-HCl respectively. Half saturation $[S]_{0.5}$ and V_{max} remain unchanged. Pyruvate kinase in Tris-maleate is slightly more stable to heating at 60°C than in Tris-HCl. Incubation of the enzyme in Tris-maleate for one h with high concentrations of dithiotreitol restores the positive homotropic interactions.

3. It is proposed, that the abnormalities of the pyruvate kinase of some patients with acquired pyruvate kinase deficiency, obtained from a study in Tris-maleate, may partly be induced by the buffer itself.

Introduction

A few years ago, van Berkel et al. proposed that human erythrocyte pyruvate kinase deficiency could be a consequence of an increased oxidized glutathione level in the red blood cell [1]. He described a class of patients, in which the pyruvate kinase abnormality was characterized among other things by an increased affinity for phosphoenol-pyruvate and a loss of positive homotropic interactions towards this substrate. Incubation of the enzyme from these patients with 10 mmol/l 2-mercaptoethanol normalized its kinetics [2]. Staal et

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al. demonstrated that one of these patients also had a decreased glutathione reductase activity. Administration of 36 mg riboflavin daily for six months restored the glutathione reductase activity, and normalized the pyruvate kinase kinetics [3]. Both studies [2,3] were performed using the buffer Tris-maleate. It is not clear why in the past this buffer was used.

On the other hand, Blume et al. [7] reported GSSG to be in the normal range in pyruvate kinase deficient erythrocytes. They stated that physiological concentrations of GSSG failed to alter the stability or kinetics of human erythrocyte pyruvate kinase [7]. They also reported that treatment over a four to six month period of six pyruvate kinase deficient patients with riboflavin, produced no significant hematologic or enzymologic changes in the variants investigated [8]. These results are consistent with our previous report, in which we show, that intra-erythrocyte oxidation of GSH to GSSG by diamide causes no changes in V_{max} or Hill coefficient of human erythrocyte pyruvate kinase [9].

In this work we show that maleic acid is an oxidizing agent, and may induce changes in human erythrocyte pyruvate kinase which are in some respect similar to these also described for the pyruvate kinase of some patients with pyruvate kinase deficiency [2,3].

Materials and methods

NADH (disodium salt), ADP (disodium salt), phospho*enol*pyruvate (tricyclohexyl-ammonium salt) and lactate dehydrogenase (rabbit muscle) were purchased from Boehringer, Mannheim, F.R.G. DEAE-Sephadex was from Pharmacia, Sweden. Maleic acid and dithiotreitol were from Baker, Deventer, Holland. All reagents were of the highest purity available. Pyruvate kinase was purified from fresh human blood by the method of Staal et al. [10], except that during the procedure 2-mercaptoethanol was omitted. The final precipitate was dissolved in 0.2 mol/l Tris-HCl or Tris-maleate, pH 8.2.

Enzyme activity was measured by the method of Bücher and Pfleiderer [11]. The reaction mixture contained in a final volume of 1 ml: 0.2 mol/l Tris-HCl or Tris-maleate pH 8.2 at 25°C; 65 mmol/l KCl; 20 mmol/l $MgSO_4$; 0.1 mg lactate dehydrogenase; 0.1 mmol/l NADH and 2 mmol/l ADP. Phospho*enol*pyruvate was in the range of 0.1—10.0 mmol/l. The reaction was started by the addition of enzyme. Initial rates were monitored on a Beckman Acta double beam spectrophotometer. Free -SH-groups were determined by the method of Ellman [12]. Thermostability of the semipurified enzymes was determined in Tris-HCl and Tris-maleate by heating for 1 min at 60°C [10]. The protein concentration was kept at 1 mg/ml by adding bovine serum albumin to the mixture.

$$R{-}SH + HOOC{-}CH{=}CH{-}COOH \rightleftharpoons HOOC{-}CH(S{-}R){-}CH_2{-}COOH \qquad (1)$$

Results

Table I shows the influence of maleic acid upon the free -SH-groups of 2-mercaptoethanol. In Tris-HCl 2-mercaptoethanol is a stable compound,

TABLE I

INFLUENCE OF MALEIC ACID UPON FREE -SH GROUPS OF 2-MERCAPTOETHANOL

0.2 mol/l Tris-HCl, pH 8.2; 1 mmol/l 2-mercaptoethanol		0.2 mol/l Tris-maleate, pH 8.2; 1 mmol/l 2-mercaptoethanol	
Time (min)	-SH (mM)	Time (min)	-SH (mM)
0	0.98	0	1.03
30	0.96	30	0.50
60	1.03	60	0.28
90	0.92	90	0.14
120	0.92	120	0.06
150	0.93	150	0.04

whereas in Tris-maleate (0.2 mol/l, pH 8.2, room temperature) 1 mmol/l 2-mercaptoethanol has a half life of approximately 30 min. This fully agrees with the results of Morgan and Friedman [13], who demonstrated an addition reaction of several thiol compounds with maleic acid, according to Equation 1.

Fig. 1 shows the activity of pyruvate kinase in Tris-HCl and Tris-maleate respectively, as a function of the phospho*enol*pyruvate concentration. In 0.2 mol/l Tris-HCl pH 8.2, it shows a sigmoidal response to phospho*enol*pyruvate, with half-saturation at 0.7 mol/l (±S.D. = 0.1, n = 10 observations), and a Hill coefficient n = 1.7 (±S.D. = 0.1, n = 10 observations) is obtained. On the other hand, in 0.2 mol/h Tris-maleate pH 8.2 the kinetics approach Michaelis-Menten kinetics with a half-saturation at 0.6 mmol/l (0.5—0.8) and a Hill coefficient n = 1.1 (1.0—1.2) (average and extreme values from five experiments). V_{max} remains the same.

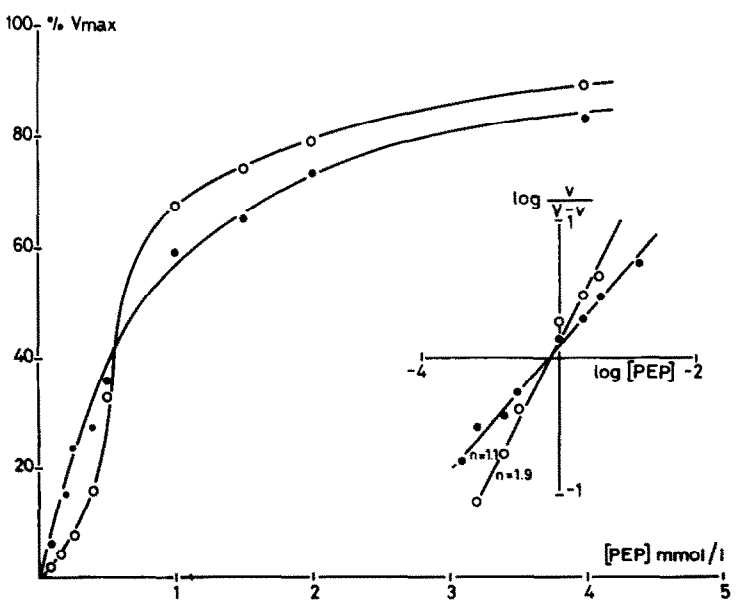

Fig. 1. v vs. phospho*enol*pyruvate of human erythrocyte pyruvate kinase, dissolved and measured in 0.2 M Tris-HCl pH 8.2 and 0.2 mol/l Tris-maleate, pH 8.2. ●———●, Tris-maleate; ○———○, Tris-HCl.

Heating of the enzyme for 1 min at 60°C causes only little loss of activity (about 10%) in Tris-maleate; in Tris-HCl this value is approx. 35%.

Incubation of a sample in Tris-maleate for two h on ice with 10 mmol/l dithiotreitol, normalizes the enzyme; i.e. a Hill coefficient of 1.6—1.8 is obtained. Incubation of a sample in Tris-HCl with 10 mmol/l dithiotreitol, causes no changes in the kinetic properties.

Discussion

This paper describes two experimental facts. In the first place it shows the reactivity of 2-mercaptoethanol towards maleic acid, a phenomenon described as early as 1938, but overlooked by many contemporary authors. In the literature on pyruvate kinase at least 16 studies have appeared utilizing maleic acid, most of them in combination with a thiol compound, to keep the enzyme in a reduced form [2,3,14—27].

Secondly it describes the influence of maleic acid upon human erythrocyte pyruvate kinase. We consistently find a loss of positive homotropic interactions for the enzyme dissolved and quantitated in Tris-maleate as compared to Tris-HCl, whereas V_{max} and $[S]_{0.5}$ for the substrate phospho*enol*pyruvate remain unchanged.

Van Berkel et al. [2] describe a class of patients in which the pyruvate kinase is characterized by an increased affinity for phospho*enol*pyruvate, and a loss of positive homotropic interactions. Their data were collected from a study in Tris-maleate. Therefore it is quite possible that the changes in Hill coefficient they observed are due to the use of Tris-maleate as buffer. This is further supported by the treatment of the enzyme in Tris-maleate with dithiotreitol: this treatment normalized the Hill coefficient. The same is found for the class of acquired pyruvate kinase deficiency mentioned above.

With respect to the heat stability, pyruvate kinase from the patients differs from the normal enzyme in Tris-maleate. The latter shows, under the conditions used, only a 10% decrease in activity, while the enzyme from the patient looses approx. 70% [3]. So that abnormal properties reported for the patients' pyruvate kinase [2,3] may only partly be caused by the use of Tris-maleate buffer.

In conclusion, we consider that maleic acid is unsuitable for the use in a study of acquired pyruvate kinase deficiency, and should not be used in the study of enzymes in general.

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References

1 Van Berkel, T.J.C., Koster, J.F. and Staal, G.E.J. (1973) Biochim. Biophys. Acta 321, 496—502
2 Van Berkel, T.J.C., Staal, G.E.J., Koster, J.F. and Nijessen, J.G. (1974) Biochim. Biophys. Acta 334, 361—367

3 Staal, G.E.J., Van Berkel, T.J.C., Nijessen, J.G. and Koster, J.F. (1975) Clin. Chim. Acta 60, 323—327
4 Kahn, A., Marie, J., Bernard, J., Cottreau, D. and Boivin, P. (1976) Clin. Chim. Acta 71, 379—387
5 Arnold, H., Blume, K.G., Löhr, G.W., Boulard, M. and Najean, Y. (1974) Clin. Chim. Acta 57, 187—189
6 Zanella, A., Rebulla, P., Giovanetti, A.M., Curzio, M., Pescarmona, G.P. and Sirchia, G. (1976) Br. J. Haematol. 32, 373—385
7 Blume, K.G., Arnold, H., Löhr, G.W. and Schulz, G. (1974) Biochim. Biophys. Acta 370, 601—604
8 Blume, K.G., Arnold, H., Hasslinger, K. and Löhr, G.W. (1976) Clin. Chim. Acta 71, 331—334
9 Sprengers, E.D., Van Berkel, T.J.C., Koster, J.F. and Staal, G.E.J. (1977) Clin. Chim. Acta 80, 495—502
10 Staal, G.E.J., Koster, J.F., Kamp, H., Van Milligen-Boersma, L. and Veeger, C. (1971) Biochim. Biophys. Acta 227, 86—96
11 Bücher, T. and Pfleiderer, G. (1965) Methods Enzymol. 1, 435—440
12 Ellman, G.L. (1959) Archives Biochem. Biophys. 82, 70—77
13 Morgan, E.J. and Friedmann, E. (1938) Biochem. J. 32, 733—742
14 Staal, G.E.J., Koster, J.F. and Nijessen, J.G. (1972) Biochim. Biophys. Acta 258, 685—687
15 Staal, G.E.J., Koster, J.F. and Van Milligen-Boersma, L. (1970) Biochim. Biophys. Acta 220, 613—616
16 Koster, J.F., Staal, G.E.J. and Van Milligen-Boersma, L. (1971) Biochim. Biophys. Acta 236, 362—365
17 Rozengurt, E., Jiménez de Asúa, L. and Carminatti, H. (1969) J. Biol. Chem. 244, 3142—3147
18 Carminatti, H., Jiménez de Asúa, L., Redondo, E., Passeron, S. and Rosengurt, E. (1968) J. Biol. Chem. 243, 3051—3056
19 Costa, L., Jiménez de Asúa, L., Rosengurt, E., Bade, E.G. and Carminatti, H. (1972) Biochim. Biophys. Acta 289, 128—136
20 Carminatti, H., Rosengurt, E. and Jiménez de Asúa, L. (1969) FEBS Lett. 4, 307—310
21 Jiménez de Asúa, L., Rosengurt, E. and Carminatti, H. (1970) J. Biol. Chem. 245, 3901—3905
22 Prager, M.D. and Whigham, W.R. (1966) Biochim. Biophys. Acta 132, 181—184
23 Farina, F.A., Shatton, J.B., Morris, H.P. and Weinhouse, S. (1974) Cancer Res. 34, 1439—1446
24 Crow, V.L. and Pritchard, G.G. (1976) Biochim. Biophys. Acta 438, 90—101
25 Sparman, G., Schulz, J. and Hofmann, E. (1973) FEBS Lett. 36, 305—308
26 Borgman, A.I. and Moon, Th.W. (1976) J. Comp. Physiol. 107, 185—199
27 Moon, Th.W. and Borgmann, A.I. (1976) J. Comp. Physiol. 107, 201—210