INVESTIGATIONS ON THE CAUSES FOR THE PSE-SYNDROME - COMPARATIVE STUDIES ON GLYCOLYTIC ENZYMES FROM PSE- AND NORMAL PIG MUSCLES

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Background

The main biochemical attribute of PSE-prone muscles is an accelerated metabolism of carbohydrates *post mortem* (Briskey et al., 1966) which has been tried to be explained in several ways: Hormonal deregulation of energy turnover processes (Altrogge et al., 1980); deregulation of the Ca^{2+}-concentration in the muscle-cell (Rock and Kozak-Reiss, 1987); ATP-consuming active membrane transport (Kim et al., 1984) futile cycling in the glycolytic metabolism (Clark et al., 1973). Increased activities of lipases and proteases are assumed to be the reason for structural alterations of membranes and myofibrillar proteins (Cheah et al., 1986). Other PSE-researchers speculated about uncoupling of ATP-formation in mitochondria (Eikelenboom and van den Bergh, 1973).

There are several authors pointing out differences between enzymes of the glycogenolytic and glycolytic pathways from PSE- and normal muscles. One of these enzymes is glycogen-phosphorylase (GP) (EC 2.4.1.1.) catalysing the degradation of glycogen which is regulated by allosteric mechanisms and phosphorylation. Ono et al. (1977) found an enhanced GP a (phosphorylated form) activity in PSE-tissue. Schwägele and Honikel (1989) determined a higher total activity (GP a and GP b) in PSE-prone muscles, whereas Ensinger et al. (1982) could not find differences in the activities of GP a and GP b in muscles of normal and PSE-animals. Studies on pyruvate kinase (PK) (EC 2.7.1.40.) had revealed characteristic differences in specific and total activity of the enzyme from normal and PSE-muscles (Schwägele et al., 1994).

Objectives

The latter mentioned results concerning the carbohydrate metabolism were obtained with crude extracts from muscle homogenates. Here we report about the purification and characterization of glycogen phosphorylase and pyruvate kinase from normal and PSE-prone muscle.

Materials and Methods

For the differentiation between normal and PSE-prone German Landrace pigs the halothane test (up to four times at intervals of 2 or 3 days) in the live animal at a weight of about 30 kg as well as the measurement of pH in the muscle (45 min *post mortem*) were used. The enzymes were isolated from M. longissimus dorsi thoracis et lumborum of 5 normal and 5 PSE-animals with a slaughter weight of about 100 kg immediately after slaughter.

The activity of GP was determined according to Bergmeyer (1970) in the direction of glycogen degradation and according to Cori et al. (1950) in the direction of glycogen synthesis. GP b was phosphorylated with phosphorylase kinase from rabbit muscle according to a procedure of Berndt and Rösen (1984).

The activity of pyruvate kinase (PK) was determined by a coupled enzymatic assay designed by Scopes (1977). PK was phosphorylated in vitro according to Hjelmquist et al. (1974). Analytical isoelectric focusing (IEF) was performed according to Frey et al. (1986). Phosphorylation of PK from normal muscle and isoform 1 was performed by means of the catalytic subunit of cAMP-dependent protein kinase from pig heart. The phosphorylation mixture (200 µl) contained 5 mM Tris/Mes, pH 6.5, 50 µM ATP, 5 mM Mg-acetate, 20 mM KCl, 200 µg PK and 4 units protein kinase. Incubation was at 30°C over night. The amount of phosphate incorporated during in vitro phosphorylation was determined from the incorporation of radioactive phosphate into the protein. The assay contained 5 mM Tris/Mes, pH 6.5, 5 mM Mg-acetate, 20 mM KCl, 0,1 mM γ-^{32}P-ATP (8 µCi/mMol), 81 µg of isoenzyme 1, and 8.5 U/ml cAMP-dependent protein kinase. Following incubation at 30°C for 18 h, aliquots of 40 µl were withdrawn from each assay and spotted on filter discs, which were incubated for 5 min in 5% trichloroacetic acid (TCA), washed three times for 5 min in 3 ml 5% TCA, dried at 80°C and transferred to a szintillation tube. The precipitated radioactivity was determined by liquid scintillation counting. Control assays without pyruvate kinase were performed in order to determine the background of nonspecific binding of radioactive ATP to the filter discs.

Results

Isoform patterns of GP and PK

Isoform patterns of the enzymes isolated from normal and PSE-muscles were studied by analytical IEF on horizontal polyacrylamide gels: The isoelectric points of at least five GP isoenzymes from normal and PSE-animals appeared in a narrow range between 6,28 and 6,53. PK from PSE-muscle shows three isoforms, whereas for the enzyme from normal muscle only two isoform bands are observed. All isoforms of the enzyme fall into the pH-range between 8.1 and 8.4. Isoform 3 which is observed only in the preparation from PSE-muscle has the lowest pI. Similar results were obtained for GP and PK in the presence of protease inhibitors showing that the number of bands on IEF gels is not due to proteolytic artefacts produced during purification (Lopez Buesa et al., 1995)

Steady state parameters of GP and PK

In contrast to GP b the phosphorylated form a is active in the absence of AMP. The affinity of GP a to glycogen and glucose-1-phosphate is higher than in the case of GP b. In interaction with P_i, glycogen and glucose-1-phosphate GP a showed the characteristics of a Michaelis-Menten kinetic. The optimum pH for both forms of GP isolated from normal as well as PSE-meat is around 7,0.

The apparent K_m- and V_{max}-values for the substrates glycogen and glucose-1-phosphate are similar to those as published in the literature. Taking these values in consideration no significant differences were found in comparison of GP b from normal and PSE-meat (table 1).

Substrate	P_i	AMP	PSE-muscle		normal muscle	
	[mM]	[mM]	K_m [mM]	V_{max} [U/ml]	K_m [mM]	V_{max} [U/ml]
Glycogen	75	0,5	0,26	28,6	0,22	26,7
	20	0,5	1,0	26,3	1,1	22,6
	5	0,5	2,2	9,1	2,9	9,1
G1P		0,1	58	53	51	55
		0,2	13	53	17	55
		1,0	8	53	7	55

Table 1: Apparent K_m- and V_{max}-values for the interaction of GP b with glucose-1-phosphate and glycogen.

Similar results were obtained for GP a isolated from normal and PSE-meat at variable concentrations of glycogen, G1P and P_i (table 2). A typical Michaelis-Menten behaviour of PK towards ADP and PEP was observed. No differences in the kinetic parameters were detectable for the utilization of ADP comparing the enzyme from normal and PSE- muscle. Significant differences, however, were observed for PEP

utilization. The enzyme from PSE-muscle has a five-fold lower K_m value and two-fold higher turnover number for PEP as compared to the enzyme from normal muscle which results in a tenfold higher efficiency of PEP utilization for the enzyme from PSE-muscles (table 3).

Variable substrate concentr.	Constant substrate concentr.	PSE-muscle K_m [mM]	PSE-muscle V_{max} [µMol/min ml]	Normal muscle K_m [mM]	Normal muscle V_{max} [µMol/min ml]
G1P	5 mM Glyc.	5,7	45,1	5,2	40,3
Glycogen	3 mM Pi	*0,20*	8,7	*0,19*	7,1
	20 mM Pi	*0,20*	15,4	*0,19*	14,5
Pi	0,14 mM Glyc.	5,3	5,0	5,1	5,2
	0,20 mM Glyc.	4,5	5,6	4,3	5,6
	0,55 mM Glyc.	3,5	7,1	3,1	6,5

Table 2: K_m- and V_{max}-values of GP a isolated from PSE- and normal muscle in interaction with G1P, glycogen and P_i. For the interaction of GP a with glycogen and P_i the two parameters were determined under different concentrations of these two substrates in the presence of 0,5 mM AMP. The reverse reaction with G1P was performed at a constant glycogen concentration (0,5 mM) in absence of AMP.

Kinetic constants		Pyruvate kinase from PSE-muscle	Pyruvate kinase from normal muscle
K_m	[µM]	17	91
k_{cat}	[s^{-1}]	1075	445
k_{cat}/K_m	[$\mu M^{-1} s^{-1}$]	63	4,9

Table 3: Steady state parameters of PK from muscle of normal and PSE-pigs. pH of test mixture = 7.0.

Kinetic constants	Isoform 1	Isoform 2	Isoform 3
SpAc [U/mg]	271	597	2890
K_m [µM]	142	67	5
k_{cat} [s^{-1}]	459	798	1505
k_{cat}/K_m [$\mu M^{-1} s^{-1}$]	3,2	12	300

Table 4: Kinetic constants of isolated isoforms of pyruvate kinase from PSE-muscle. pH of test mixture = 7.0. SpAc = specific activity.

Isolation and kinetic characterization of PK isoforms

The various PK isoforms from normal and PSE-muscles could be quantitatively isolated from gels of preparative IEF and the steady state parameters were measured. Isoform 1 has the lowest specific activity but is present at the highest amount in both enzyme preparations. Isoforms 1 and 2 do not differ in the specific activity when isolated from normal or PSE-muscle. Isoform 3, which is present only in pyruvate kinase from PSE-muscle, has a more than ten-fold higher specific activity compared to isoform 1. The presence of isoform 3 seems to be responsible for the high specific activity of pyruvate kinase from PSE-muscle.

The differences in the properties of the various isoforms are highlighted best by the comparison of the k_{cat}/K_m values for utilization of PEP. Isoform 3 has a nearly 100-fold higher k_{cat}/K_m value as compared to isoform 1, whereas isoform 2 is about 4 times more efficient than isoform 1 (table 4).

In vitro phosphorylation of isoform 1 and PK from normal muscle

The influence of phosphorylation and dephosphorylation reactions on the activity and electrophoretic behaviour of pyruvate kinase was studies. Since isoform 3 had the lowest pI of the isoforms and possibly represented a higher phosphorylated form we tried first to phosphorylate pyruvate kinase from normal muscle in vitro by incubation with cAMP-dependent protein kinase. Upon isoelectric focusing of the reaction products a third band is detectable that migrates at the position of isoform 3. Possibly isoforms 2 and 3 arise from isoform 1 by protein phosphorylation. Treatment of the enzyme from PSE-muscle with alkaline phosphatase leads to a decrease in intensity of the band corresponding to isoform 3. At the same time the intensity of the bands corresponding to isoforms 1 and 2 increased.

Discussion and Conclusions

The turnover of ATP is increased and glycolysis is accelerated in PSE-muscles. Comparative studies on the activity, structure and posttranslational modification of GP and PK, key enzymes in glycolysis, which may directly participate in the metabolic deviations characteristic for the PSE-syndrome, were performed.

Striking differences in the enzymatic properties of isolated PK from normal and PSE-muscle were detectable. The higher catalytic efficiency of PK from PSE-muscle is due to a higher affinity for phosphoenol pyruvate as expressed by a lower K_m. The enzyme from PSE-muscle contains 3 isoforms while only two isoforms are detectable in the preparation of the enzyme from normal muscle. Isoform 3 which is specific to the enzyme from PSE- muscle is the most active of the three isoforms being more than 100-fold more efficient than isoform 1. In vitro experiments on the phosphorylation and dephosphorylation of total enzyme and of isolated isoform 1 strongly suggest that isoform 3, which is more active under acidic conditions, arises from isoforms 1 and/or 2 by protein phosphorylation. At present we do not know whether the PSE-specific isoform 3 is present already in the muscles of live animals or whether it is generated only under *post mortem* conditions.

GP isolated from PSE-muscle did not differ in a characteristic way from the enzyme isolated from normal muscle. From the experiments reported it appears, that GP does not permanently exist in different active isoenzymes in dependency upon PSE- and normal pigs besides the existing phosphorylated GP a and not phosphorylated GP b forms. Unlike PK in which an additional more active and stable phosphorylated isoenzyme could be proved, GP is controlled in pig muscles in a similar way, but this control is continuously switched on and off according to the need of muscle, i.e. ATP consumption rate by metabolism. Thus the high energy turnover (ATP-turnover) in muscles of PSE-pigs is possible by the turnover rate of GP in both existing forms.

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