JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Postmortem Changes in Pork Muscle Protein Phosphorylation in Relation to the RN Genotype

René Lametsch,[*,†] Martin R. Larsen,[‡] Birgitta Essén-Gustavsson,[∥] Marianne Jensen-Waern,[∥] Kerstin Lundström,[§] and Gunilla Lindahl[†,§]

[†]Department of Food Science, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

[‡]Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense, Denmark

[§]Department of Food Science, Swedish University of Agricultural Sciences, P.O. Box 7051, SE-750 07 Uppsala, Sweden

[∥]Department of Clinical Sciences, Swedish University of Agricultural Sciences, P.O. Box 7051, SE-750 07 Uppsala, Sweden

ABSTRACT: Postmortem changes in pork muscle protein phosphorylation in relation to the RN^- genotype were investigated using one-dimensional gel electrophoresis and a phosphor specific staining. The phosphorylation levels of several protein bands were found to be affected by the RN^- genotype and to change during postmortem development. Glycogen phosphorylase, phosphofructokinase, and pyruvate kinase were found in protein bands affected by the RN^- genotype, and the phosphorylation profile indicates that part of the increased rate and extended pH decline of the RN^- genotype could be a consequence of phosphorylation of these key enzymes during the postmortem metabolism. The results illustrate that the protein phosphorylation level of the muscle proteins could be interpreted as a global metabolic fingerprint containing information about the activity status of the enzymes in the postmortem metabolism.

KEYWORDS: Protein phosphorylation, pork, RN^- genotype, postmortem metabolism, glycolysis, glycogenolysis

INTRODUCTION

The postmortem (PM) metabolism which takes place during the conversion of muscle to meat has major impact on the final meat quality.[1] The termination of the blood circulation at death initiates a complex series of changes in muscular tissue. Initially the oxygen supply stops, which results in change of energy metabolism from aerobic oxidative metabolism to anaerobic glycolytic metabolism. This results in accumulation of lactate in the muscle PM and pH drops from about 7.2 to around 5.3−5.7, depending on genotype, muscle type, feeding, stress, slaughter process, and species.[2]

Because of the stoppage of circulation of blood, glucose has to be generated through breakdown of glycogen involving glycogen debranching enzyme, glycogen phosphorylase, and phosphoglucomutase, where glycogen phosphorylase is the rate limiting enzyme.[1] In the glycolysis hexokinase, phosphofructosekinase (PFK) and pyruvate kinase catalyze the essential irreversible reactions and are also the rate limiting enzymes. Several studies have investigated the influence of these rate limiting enzymes on the PM metabolism in animals used for meat production.[3,4]

PFK is the key glycolytic enzyme in the living muscle with the most sophisticated regulatory mechanisms. This enzyme is regulated by pH, a variety of ligands including its substrates, reaction products, various metabolites such as AMP, ATP, fructose 2,6 biphosphate (F2,6BP), citrates, and lactate.[5,6] Recently, it was found that lactate favors the dissociation of PFK tetramers downregulating the enzyme and glycolysis. However, this effect can be prevented by tetramer-stabilizing conditions such as the presence of F2,6BP and phosphorylation of the enzyme.[6] These results indicate that phosphorylation of PFK and the content of F2,6BP could be very important in the regulation of the PM metabolism because of the high level of lactate.

The AMP-activated protein kinase (AMPK) is involved in the regulation of the energy metabolism in the skeletal muscle,[7] and it has also been shown that the activity of AMPK is related to the PM metabolism.[8,9] In pigs, a dominant mutation has been identified in the gene coding for AMPK (RN^- allele), causing a marked increase in the muscle glycogen content.[10] It is well established that the RN^- allele leads to an extended pH decline, resulting in a decrease in the water holding capacity (WHC).[1] Furthermore, it has been revealed that the RN^- genotype has a higher basal AMPK activity and diminished AMP dependence compared to wild-type.[11] However, the precise mechanism behind the extended pH decline in pigs with the RN^- genotype is still unclear[1]

Protein phoshorylation is a key regulator of biological processes and is also known to be involved in the regulation of the energy metabolism in the cell.[12] In a recent study, it was found that the sarcoplasmic protein are phosphorylated PM and that the protein phosphorylation is related to the rate of the pH decline PM.[13] The aim of this study is to investigate the PM changes in protein phosphorylation in the longissimus dorsi muscle from pigs with and without the RN^- allele using a combination of one-dimensional (1D) gel electrophoresis and phosphor specific staining to gain further knowledge about the metabolic mechanism that causes the extended pH decline in pigs with the RN^- genotype.

Received: May 16, 2011
Accepted: September 29, 2011
Revised: September 19, 2011
Published: September 29, 2011

ACS Publications © 2011 American Chemical Society

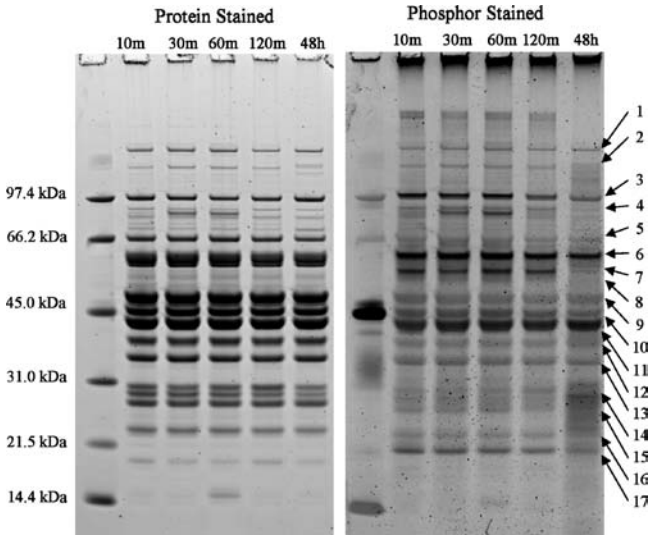

Figure 1. SDS-PAGE of sarcoplasmic protein samples from one animal at five different time points PM. The same gel is first stained with a phosphor specific staining and then with a protein specific staining.

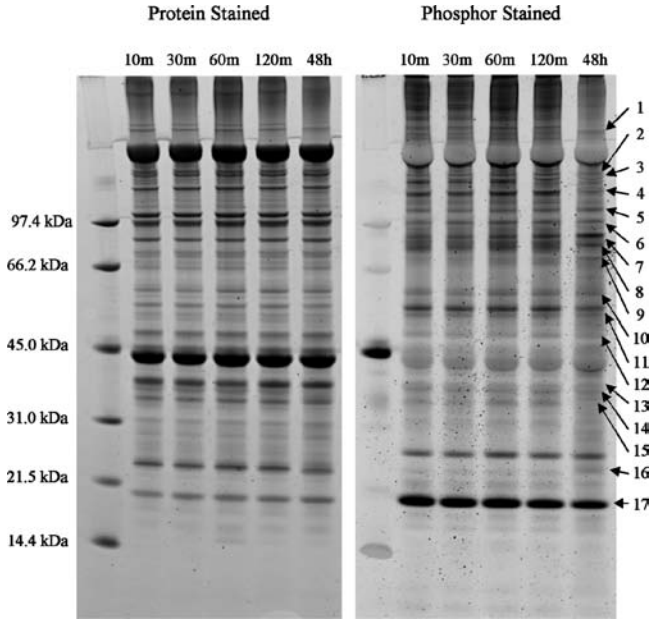

Figure 2. SDS-PAGE of myofibrillar protein samples from one animal at five different time points PM. The same gel is first stained with a phosphor specific staining and then with a protein specific staining.

MATERIALS AND METHODS

1. Sampling. Eight clinically healthy pigs (Yorkshire/Swedish Landrace x Hampshire), four with RN^- genotype and four wild-type shown by DNA analysis on the blood made according to Milan et al.[10] were used. The pigs were housed in pens (four pigs/pen) with straw as bedding. They were fed a commercial finisher diet ad libitum and had free access to water. The pigs were killed with a captive bolt and exsanguinated when they weighed 67 ± 13 kg. Samples of LD were collected at different time points PM, immediately frozen in liquid nitrogen, and stored at −80 °C until analysis. The first sample was taken within 10 min PM, and then samples were taken at 30 min, 1, 2, and 48 h PM. The experiment was carried out according to the Swedish Inspectorate of Animal Experimentation guidelines.

2. Sample Preparation. One gram of frozen muscle sample was homogenized in 6 mL of 0.05 M KCl, 5 mM iodoacetamide, Complete (Roche, Hvidovre, Denmark), and PhosStop (Roche, Hvidovre, Denmark), 2 × 30 s (9500 rpm) and 2 × 30 s (13000 rpm), using a Ultra-Turrax T25 equipped with a S25N-18 G dispersing element (Ika Labortechnik, Staufen, Germany). Between each homogenization step, the samples were incubated 30s on ice. The pH was determined in the suspension and neutralized by adding 1 mL of 0.7 M Tris (pH 8.3) and 70 mM DTT. The sample was centrifuged for 20 min at 15000*g* (4 °C). The supernatant was removed and stored at −80 °C (sarcoplasmic fraction). Then 25 mL of SDS-buffer (5%) was added to the pellet and then homogenized for 30 s at 9500 rpm using an Ultra-Turrax T25 equipped with a S25N-18 G dispersing element (Ika Labortechnik, Staufen, Germany), followed by 20 min incubation in a water bath at 80 °C. The dissolved pellet was stored at 4 °C (myofibrillar fraction). The protein concentration was determined by measuring the absorption at 280 nm using a spectrometer (Helios Omega, Thermo Scientific, US). The protein concentration was estimated by using the extinction coefficient ABS_{280} 1 = 1 mg/mL.

3. Gel Electrophoresis. The sarcoplasmic and the myofibrillar fractions were analyzed using SDS-PAGE according to the manufacturer (4−12% Bis-Tris NuPage gels, 15 wells, with Mes running buffer, Invitrogen, Taastrup, Denmark). For the sarcoplasmic fraction, 7.5 μg protein was loaded in each lane, and for the myofibrillar fraction, 3 μg was loaded. Samples from the different time points PM from two animals were loaded on each gel, one with RN^- genotype and one wild-type. Each gel was repeated three times. Staining of phosphorproteins were made with Pro-Q Diamond (Invitrogen, Taastrup, Denmark), followed by a total protein staining made with Sypro Ruby (Invitrogen, Taastrup, Denmark). The staining was made according to the manufacturer. Detection of Pro-Q Diamond and Sypro Ruby staining were made using a Typhoon scanner (GE-Healthcare, Hilleroed, Denmark) using the following settings, respectively: Laser, green 532, 500 V, 100 μm; filter, 580 BP30; and laser, green 532, 470 V, 100 μm; filter, 610 BP30. Image analysis was made using Phoretix 1D, V2003.2 (Nonlinear Dynamics, Newcastle, UK). The phosphorylation level of the individual protein bands were defined as the ratio between the intensity of the Pro-Q Diamond staining and the Sypro Ruby staining.

4. Protein Identification. Protein bands of interest were selected for protein identification. The protein identifications were made according to Lametsch et al.[14] The protein bands were excised from Coomassie stained gels. The gel pieces were cut into approximately 1 mm^3 pieces and washed twice for 15 min, first with water and second with water/acetonitrile 1:1 (v/v). The gel particles were then washed in acetonitrile to dehydrate the gel. A volume of 10 mM dithiotreitol (DTT) in 100 mM NH_4HCO_3 to cover the gel pieces was added and the proteins were reduced for 45 min at 56 °C. After cooling, the DTT solution was replaced by the same volume of 55 mM iodoacetamide in 100 mM NH_4HCO_3 and the reduced proteins were alkylated for 30 min in the dark. The gel pieces were then washed with water, water/acetonitrile 1:1 (v/v), and acetonitrile to dehydrate the gel. Ice-cold digestion buffer containing 12.5 ng/μL of trypsin in 50 mM NH_4HCO_3 was added to the gel pieces in a volume just sufficient to rehydrate the gel (5−10 μL). After 45 min of incubation in an ice bath, the unabsorbed digestion buffer was removed and replaced by 20 μL of 50 mM NH_4HCO_3 buffer to cover the gel pieces. The proteins were digested overnight at 37 °C.

The peptide solutions were purified on nanoscale reversed-phase columns packed with Poros R2 10 μm, (Applied Biosystems, CA) prior to mass spectrometric analysis. The peptide digest was loaded onto the columns and desalted by washing with 1% TFA. The peptides were eluted with MALDI MS matrix solution containing 5 μg/μL of cyano-4-hydroxycinnamic acid in 70% acetonitrile and 0.1% TFA directly in one droplet onto the MALDI target (Opti-TOF 384 Well MALDI Plate Inserts, Applied Biosystems, California). MS and MS/MS was carried out by the use of MALDI TOF/TOF MS using an ABI 4800 ms instrument

Table 1. Identification of Phosphorylated Proteins in the Sarcoplasmic Fraction and the Effect of Time PM and Genotype on the Phosphorylation Level[a]

			sarcoplasmic protein fraction					
no.[b]	MW[c]	MW (cal)[d]	protein ID[e]	data base ID[f]	score[g]	time	Gen	TXG
1	165	180	glycogen debranching enzyme	GDE_RABIT	132		(*)	*
2	136	128	myosin binding protein C	Q8K2 V0_MOUSE	56	***		
3	99	98	glycogen phosphorylase	Q19PY1_PIG	350	***		*** (*)
4	90	83	aconitate hydratase	1B0JA	258			
		79	transferrin	S01384	211			
		86	6-phosphofructokinase	K6PF_BOVIN	213			
5	66	71	serum albumin	ABPGS	467			
6	58	62	phosphoglucomutase-1	PGM1_RABIT	203	***		
		58	pyruvate kinase	KPYM_HUMAN	251			
7	52	52	immunoblubolin	AAA51292	81	*** (*)		
8	50	47	β-enolase	ENOB_PIG	96			
		51	elongation factor 1	EF1A2_BOVIN	90			
9	47	47	β-enolase 3	Q1KYT0_PIG	406			
		45	phosphoglycerate kinase 1	PGK1_PIG	125			
10	45	43	creatine kinase	Q45EW9_PIG	246	***		
11	43	40	fructose-bisphosphate aldolase	ALDOA_RABIT	262	*** (***)		**
12	39	36	glyceraldehyde-3-phosphate DH	DEPGG3	154	*		
13	36	37	L-lactate dehydrogenase A	DEPGLM	197			
14	29	29	phosphoglycerate mutase 2	PGAM2_BOVIN	121	***		
15	27	27	triosephosphat isomerase 1	TPIS_PIG	325	* (*)		
16	23	22	adenylate kinase	KAD1_PIG	251	***		
		17	myoglobin	MYPG	312			
17	21	19	cofilin-2	COF2_PIG	97			

[a] The stars in the brackets are results of statistical analysis of samples from 10 to 120 min (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). [b] Gel band number. [c] Estimated molecular weight from SDS-PAGE. [d] Theoretical molecular weight obtained from protein database. [e] Protein identification obtained by MS analysis. [f] Protein database ID (Swissprot or MSDB). [g] Mascot protein score.

(Applied Biosystems, CA). External mass calibration was applied using a tryptic digest of β-lactoglobolin (m/z 837.48 and 2313.26). Peak lists were generated using Data Explorer (Applied Biosystems, CA). Mascot MS/MS Ions Search (Matrix Science, http://www.matrixscience.com) was used to search for matching protein sequences within the databases Swiss-Prot (Swiss Institute of Bioinformatics, http://www.expasy.ch/sprot/) or MSDB (http://www.proteomics.leeds.ac.uk/bioinf/msdb.html). The taxonomy was restricted to mammalian. The mass tolerance was limited to 70 ppm for peptide mass fingerprinting and to 0.6 Da for peptide sequence data. Protein with significant ($p < 0.05$) Mascot protein scores were regarded as possible candidates for identification.

5. Statistical Analysis. Analysis of variance was carried out with the Statistical Analysis System version 9.1 (SAS Institute Inc., Cary, NC). The MIXED procedure was applied when calculating least-squares mean (LSM) and standard error (SE). The statistical model for the dependent variable (individual proteins) included the independent variables genotype (RN genotype and wild-type), time point PM (10, 30, 60, 120 min and 48 h), and their interaction as fixed effects and animal as random effect. The option PDIFF with Bonferroni adjustment for multiple comparisons was used to calculate P values for significant differences between LSMs. Significant differences between the genotypes at each time point PM were tested as were the differences between the time points PM within each genotype. P values ≤ 0.05 were considered significant.

RESULTS

1. Protein Phosphorylation of Sarcoplasmic and Myofibrillar Proteins. Proteins from eight pig muscle samples, sampled at different time points PM, were fractionated into a sarcoplasmic fraction and a myofibrillar fraction. The proteins from both fractions were separated using SDS-PAGE. The proteins were initially stained with a phosphor specific staining (Pro-Q diamond) followed by a fluorescent protein staining. In Figures 1 and 2, the SDS-PAGE gels of the sarcoplasmic and the myofibrillar proteins from the different time points PM from one animal are illustrated, respectively. The figures clearly show that proteins in several bands are phosphorylated. Around 20−25 protein bands are phosphorylated in both the sarcoplasmic and the myofibrillar fraction. By comparing the phosphor specific staining and the protein staining of the same gels both in Figure 1 and 2, it can be observed that the staining intensity of the different bands are dissimilar, indicating that the phosphor staining is specific. The phosphorylation level of the individual protein bands were defined as the ratio between the intensity of the phosphor staining and the protein staining. The most intense phosphor protein bands were analyzed with the use of mass spectrometry. Seventeen protein bands both from SDS-PAGE gels with sarcoplasmic and myofibrillar proteins were analyzed. The protein identifications are shown in Tables 1 and 2. Nineteen proteins were identified in the bands from the sarcoplasmic fraction and 22 proteins in the myofibrillar fraction. A total of 32 nonredundant proteins were identified in the bands from the two fractions. Nine proteins were identified in both fractions. Most of the identified protein originates from pig, however, some of the identified proteins were from other species such as bovine, rabbit,

Table 2. Identification of Phosphorylated Proteins in the Myofibrillar Fraction and the Effect of Time Postmortem and Genotype on the Phosphorylation Level[a]

			myofibrillar protein fraction					
no.[b]	MW[c]	MW (cal)[d]	protein ID[e]	data Base ID[f]	score[g]	time	gen	TXG
1	225	290	filamin 2	AAF80245	184	* (*)		
2	159	166	myomesin 2	Q723Y2_HUMAN	174	(**)		
3	153	224	myosin heavy chain 2b	Q723Y2_HUMAN	203	*		
4	132	128	myosin binding protein C	Q9TV62_PIG	102	*** (***)		(*)
5	102	104	α-actinin 3	FAHUA3	271	* (*)		
6	95	97	glycogen phosphorylase	Q19PY1_PIG	387			
7	82	86	6-phosphofructokinase	Q2HYU2_PIG	67		*	
8	73	104	*α-actinin 3*	FAHUA3	*79*	** (***)		
		224	*myosin heavy chain*	Q9TV61_PIG	*68*			
9	67	71	serum albumin precursor	ABPGS	153	*** (***)		
10	56	57	pyruvate kinase	1A49A	201	* (**)	*	
11	52	54	desmin	DESM_PIG	143	* (**)		
12	47	47	β-enolase 3	Q1KYT0_PIG	93			
13	39	36	glyceraldehyde-3-phosphate DH	DEPGG3	181	*		
		32	troponin T	TNNT3_PIG	197			
14	36	32	troponin T	TNNT3_PIG	233	* (**)		
15	31	33	adenine nucleotide translocator 1	Q9XS69_PIG	144	* (**)	(***)	
		29	phosphoglycerate mutase 2	Q32KV0_BOVIN	100			
16	24	21	MLC 1/3	A1XQT6_pig	185	***	(*)	
		21	troponin I	TNNI2_RAT	121			
		21	adenylate kinase	KAD1_HUMAN	104			
17	19	19	MLC 2	MLRS_RAT	353	***	** (*)	

[a] The stars in the brackets are results of statistical analysis of samples from 10 to 120 min (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). [b] Gel band number. [c] Estimated molecular weight from SDS-PAGE. [d] Theoretical molecular weight obtained from protein database. [e] Protein identification obtained by MS analysis. [f] Protein database ID (Swissprot or MSDB). [g] Mascot protein score.

or human, and these identifications were carefully investigated and found to be reliable homologues. Some of the protein bands contained more than one protein due to similar molecular weight and consequently comigration on the SDS-PAGE gel. If a more sensitive protein identification method had been used, more proteins would probably have been identified in each band. However, most likely are the identified proteins representing the major abundant proteins in each protein band.

The protein identifications reveal that several of the proteins in the protein bands showing phosphorylation are enzymes that are involved in the glycogenolysis or the glycolysis and most of the key enzymes in these pathways such as glycogen phosphorylase (GP), phosphofructokinase (PFK), and pyruvate kinase (PK) were identified. In total, 14 of the 32 identified proteins are involved in the glycogenolysis or the glycolysis. Most of these enzymes are found in the sarcoplasmic fraction, although seven of the glycolytic proteins were also found in the myofibrillar fraction probably because they bind hard to some of the myofibrillar proteins or because of PM protein denaturation. All of the glycolytic proteins identified in the myofibrillar fraction were also found in the sarcoplasmic fraction.

Several myofibrillar proteins were identified in the protein bands showing protein phosphorylation such as myosin binding protein C, α-actinin, desmin, myosin light chain 1/3 (MLC 1/3), and myosin light chain 2 (MLC 2). MLC 2 was especially found to be highly phosphorylated with a notably higher phosphorylation level than any of the other identified phosphorylated proteins.

2. Effect of Time. The phosphorylation level of the majority of the phosphorylated protein bands were found to change significantly during the first 48 h PM. Ten and 14 of the phosphorylated protein bands in the sarcoplasmic and the myofibrillar fraction respectively were found to change in phosphorylation PM. The profile of some of the phosphorylated protein bands are illustrated in Figure 3. These profiles show that there are both increases and decreases in the PM phosphorylation. The changes are most pronounced between 120 min and 48 h, however, 10 of the 14 myofibrillar protein bands that were found to change significantly in phosphorylation level PM were also significant if the 48 h samples were excluded from the statistical analysis (Table 2). In the sarcoplasmic fraction, 3 out of the 10 significant bands changed significantly (Table 1). This illustrates that there is already changes in protein phosphorylation in the first 2 h after slaughter.

3. Effect of Genotype. The phosphorylation level of several of the protein bands are affected by the RN^- genotype. In the sarcoplasmic fraction, three protein bands show interaction between time and genotype (Table 1). The profiles of two of these bands are illustrated in Figure 4, bands 1 and 3. Both of these bands show a significant difference between the wild and the RN^- genotype 10 min PM, whereas no difference was found at the later time points. The RN^- genotype had the highest phosphorylation ratio. Glycogen debranching enzyme and GP were identified in bands 1 and 3, respectively, both enzymes are involved in the glycogenolysis.[15] Four protein bands of the myofibrillar fraction were affected by the RN^- genotype band 7, 10, 15, and 17 (Figure 4). Two

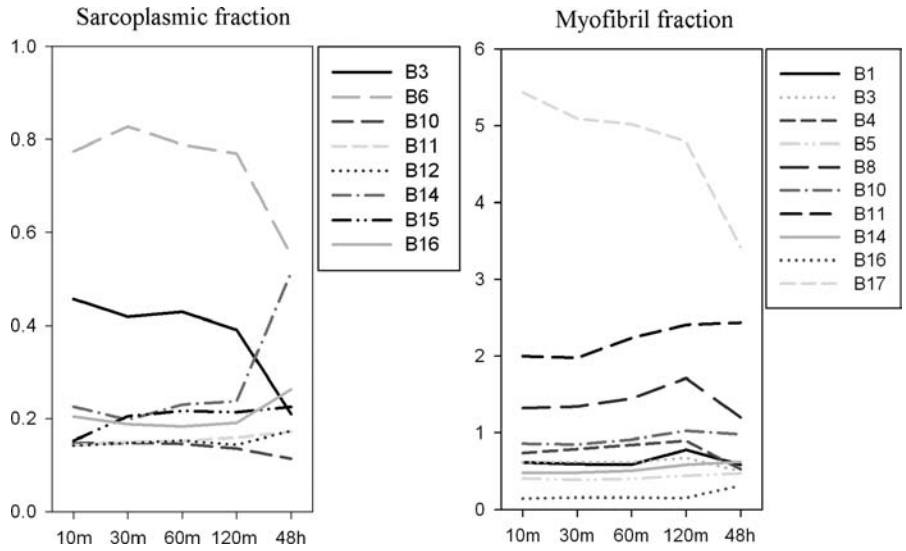

Figure 3. Postmortem changes of the phosphorylation level of the phosphorylated protein. All the illustrated bands are significantly affected by time PM ($p < 0.05$).

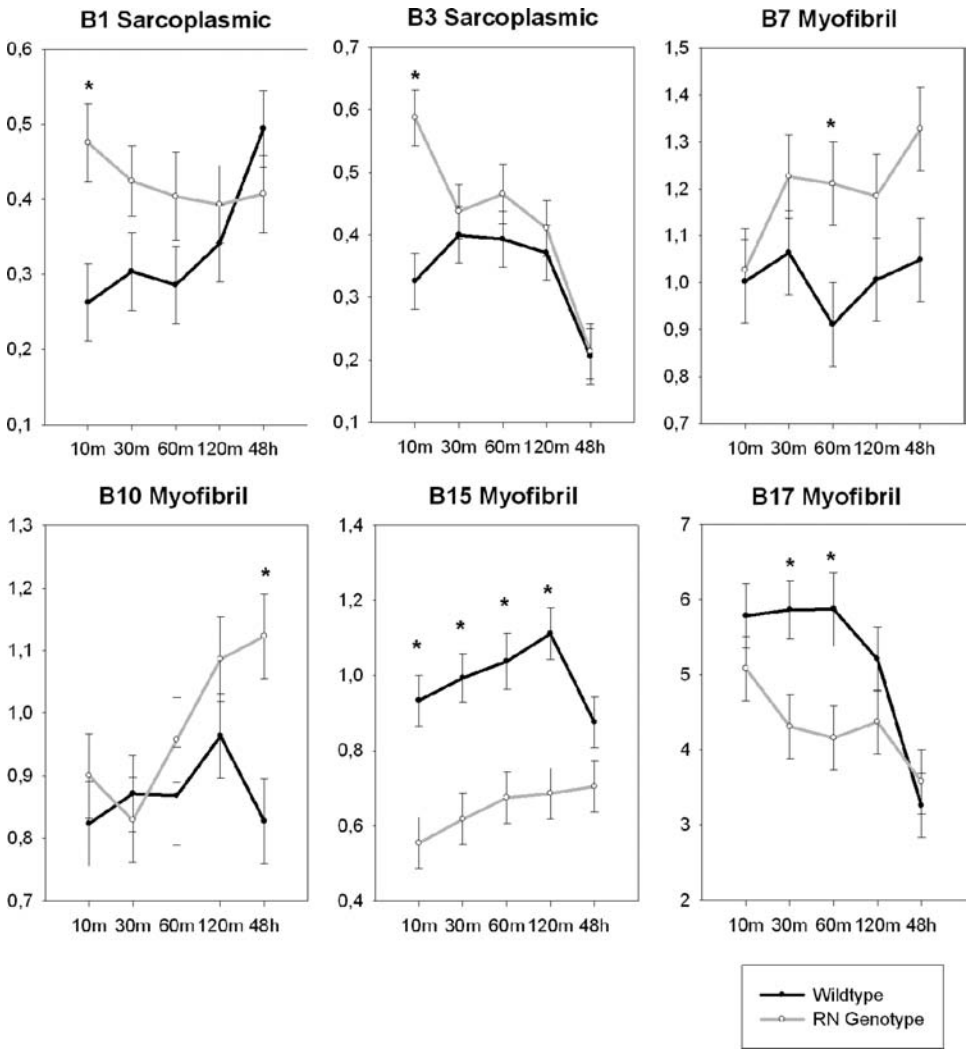

Figure 4. Effect of genotype on protein phosphorylation from both the sarcoplasmic and myofibrillar protein fractions. The stars illustrate the time point PM where there is a significant difference between the wild type and the RN^- genotype ($p < 0.05$).

of the four protein bands show a higher phosphorylation level in the RN^- genotype corresponding to PFK and PK. The most intense phosphorylated band (band 17) was also affected by genotype. MLC 2 was identified in this band.

DISCUSSION

Protein phosphorylation is one of the most common modes of regulation of protein function, and it is known to be involved in regulation of various processes including energy metabolism and protein degradation.[12,16] Protein phosphorylation is reversible and tightly controlled by kinases (phosphorylation) and phosphatases (dephosphorylation). The chemical activity of a kinase involves transferring a phosphate group from a nucleoside triphosphate (usually ATP) and covalently attaching it to an amino acids that have a free hydroxyl group such as serine, threonine, or tyrosine.[12] As the concentration of ATP decreases PM and reach a minimum at rigor mortis, it could be speculated that only limited protein phosphorylation is occurring due to substrate dilution. However, the presented results clearly show that there are significant changes in the protein phosphorylation PM in pig muscle both in the sarcoplasmic and myofibrillar. There must therefore be enough ATP present in the muscle during the first 2 h after slaughter for protein phosphorylation. Similar PM protein phosphorylation changes in pig muscle have been reported by Huang et al.[13] The changes could also be a consequence of PM protein degradation, however, the amount of protein phosphorylation is measured as a ratio between the protein amount, and the amount of phosphorylation and degradation of the phosphorylated proteins will only affect this ratio to a minor extent. The majority of the analyzed protein bands in the sarcoplasmic and myofibrillar fraction were found to change in protein phosphorylation during the first 48 h PM (Tables 1 and 2). The changes were especially pronounced between 120 min and 48 h. However, in the structural (myofibrillar) fraction most of the changes in protein phosphorylation were also significant when only the data from the first 120 min PM were included in the data analysis (Table 2).

The effect of the RN^- allele on the PM metabolism has been studied extensively, and it is well-known that RN^- mutation leads to high levels of muscle glycogen and extended pH decline PM.[17] It was revealed that the RN^- genotype was caused by a mutation in the gene coding for the AMP-activated protein kinases (AMPK), leading to a high basal AMPK activity and diminished AMP dependence.[11] However, the precise mechanism behind the relation of the mutation of the AMPK and the extended PM pH decline in muscle is still unclear. In the present study, it was found that the protein phosphorylation of several proteins were affect by the RN^- allele (Figure 4). The proteins in bands 1 and 3 in the sarcoplasmic fraction showed an interaction between time and genotype in the level of phosphorylation. Statistical analysis of the phosphorylation level at individual time points revealed that there was a significant difference 10 min PM. Here both proteins had a higher level of protein phosphorylation in the RN^- genotype. Only GP was identified in band 3 of the sarcoplasmic fraction. It is well-known that GP exist in two forms, an active phosphorylated form and a nonphosphorylated inactive form.[12] The high phosphorylation level in band 3 could therefore reflect a higher activity of GP in the RN^- genotype 10 min PM. It has been suggested that GP activity is regulated by AMPK.[8,18] Furthermore, recent studies have shown that the activity of GP is decreasing PM in pig not containing the halothane gene.[9,19] These results support the finding that the change in phosphorylation of the proteins in band 3 in the sarcoplasmic fraction is related to GP as the intensity of the protein phosphorylation is decreasing PM. In band 1, only the glycogen debranching enzyme was identified, however, regulation of glycogen debranching enzyme through phosphorylation has not been reported.

In the myofibrillar fraction, four bands were affect by the RN^- genotype. In two of the affected bands, 7 and 10 PFK and PK were identified, respectively. PFK plays a central role in the glycolysis and is regulated by a variety of metabolites.[20] Moreover, it has been found that when PFK is associated with f-actin it is stabilizing the enzyme, leading to an increased activity, and that phosphorylation of PFK increases its affinity for f-actin.[20] It has also been reported that lactate down-regulates the activity of PFK by destabilizing the enzyme but that this effect can be prevented by binding of PFK to f-actin.[6] The differences in phosphorylation intensity of band 7 in the myofibrillar fraction indicate that PFK is more phosphorylated, therefore more associated with f-actin and more resistant to lactate inhibition in the RN^- genotype, leading to a higher enzyme activity in this genotype. It has also been shown that phosphorylation of PK leads to a more acid stable isoform, which results in a more active enzyme during the PM pH decline.[21]

The changes found in protein phosphorylation of bands containing GP, PFK, and PK in this study lead to the hypothesis that the increased rate and extended pH decline of the RN^- genotype could be a consequence of phosphorylation of these key enzymes in the PM metabolism together with a high glycogen content.

Other metabolic proteins than the above-mentioned were identified where the phosphorylation levels were found to be effected by the RN^- genotype or time PM such as phosphoglucomutase-1, β-enolase, phosphoglycerate kinase 1, creatin kinase, triosephosphate isomerase 1, and adenylate kinase. Some of these enzymes were present in gel bands where more than one protein was identified, which makes it difficult to annotate the changes in phosphorylation to a specific protein. Moreover, additional proteins would probably be identified if a more sensitive method was used for protein identification also in the band where only one protein was identified. However, from most of the bands, the main phosphorylation signal is probably only from one protein. The presented results also reveal that the observed changes in protein phosphorylation are in agreement with the existing knowledge about the PM changes of the activity of the identified metabolic protein.

Especially in the myofibrillar fraction, several structural proteins were identified where the phosphorylation levels were found to be affected by the RN^- allele or time PM. The identified proteins were filamin 2, myomesin 2, myosin heavy chain, α-actinin 3, troponin T, troponin I, MLC 1/3, and MLC 2. Most of these proteins have already been described as phosphorylated and that the phosphorylation affects the activity or the functionality of the proteins.[22−26] Band 17 of the myofibrillar fraction was the most intense phosphorylated band found on the gel, and it also was revealed to be affected by both time and genotype (Figure 4). MLC 2 was the only identified protein in this band, and it has been reported to be highly phosphorylated, up to 40%.[22] The exact influence of the phosphorylation of MLC 2 on muscle contraction is not known, and the intense phosphorylation of MLC 2 in skeletal muscle is still controversial.[22] In a previous study on bovine muscle, it was found that MLC 2 was phosphorylated during PM development,[27] which is in contrast to the present results where dephosphorylation of the MLC 2

PM was observed. One explanation could be that the PM phosphorylation of MLC 2 is different in pigs and cows.

The presented results illustrate that the use of one-dimensional SDS-PAGE in combination with specific phosphor staining is a very powerful tool to investigate the PM changes of phosphorylation of muscle proteins. The results show that the protein phosphorylation of the muscle proteins could be interpreted as a global metabolic fingerprint (GMF) containing information about the activity status of most of the enzymes in the glycogenolysis and glycolytic pathways during the PM metabolism. However, it has to be emphasized that the relation between the protein phosphorylation state of the individual protein bands on the gels and the identified proteins has to be studied further as the changes in protein phosphorylation could be a consequence of other low abundant proteins that comigrate in the same protein band. Moreover, even though the activity of many enzymes are affected by protein phosphorylation, an enzyme also be phosphorylated without affecting the activity.

AUTHOR INFORMATION

Corresponding Author

*Phone: +45 3533 3483. E-mail; rla@life.ku.dk.

Funding Sources

The project was financed by the Danish Research Council (274-05-0274).

ACKNOWLEDGMENT

We acknowledge Anja Hullberg and Christina Nilsson for sampling of the meat after slaughter. Linda de Sparra Terkelsen is acknowledged for technical assistance with gel electrophoresis and image analysis.

ABBREVIATIONS USED

MS, mass spectrometry; MALDI-TOF/TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; MLC 1/3, myosin light chain 1/3; MLC 2, myosin light chain 2; AMPK, AMP-activated protein kinase; SDS-PAGE, Sodium dodesyl sulfate-polyacrylamide gel electrophoresis; GP, glycogen phosphorylase; PFK, phosphofructokinase; GMF, global metabolic fingerprint

REFERENCES

(1) Scheffler, T. L.; Gerrard, D. E. Mechanisms controlling pork quality development. The biochemistry controlling postmortem energy metabolism. *Meat Sci.* **2007**, *77*, 7–16.

(2) Huff-Lonergan, E.; Lonergan, S. M. Mechanisms of water-holding capacity of meat. The role of postmortem biochemical and structural changes. *Meat Sci.* **2005**, *71*, 194–204.

(3) Rhoades, R. D.; King, D. A.; Jenschke, B. E.; Behrends, J. M.; Hively, T. S.; Smith, S. B. Postmortem regulation of glycolysis by 6-phosphofructokinase in bovine M-sternocephalicus pars mandibularis. *Meat Sci.* **2005**, *70*, 621–626.

(4) Stephens, J. W.; Dikeman, M. E.; Unruh, J. A.; Haub, M. D.; Tokach, M. D.; Dritz, S. S. Effects of oral administration of sodium citrate or acetate to pigs on blood parameters, postmortem glycolysis, muscle pH decline, and quality attributes of pork. *J. Anim. Sci.* **2008**, *86*, 1669–1677.

(5) Fothergillgilmore, L. A.; Michels, P. A. M. Evolution of Glycolysis. *Prog. Biophys. Mol. Biol.* **1993**, *59*, 105–235.

(6) Leite, T. C.; Da Silva, D.; Coelho, R. G.; Zancan, P.; Sola-Penna, M. Lactate favours the dissociation of skeletal muscle 6-phosphofructo-1-kinase tetramers down-regulating the enzyme and muscle glycolysis. *Biochem. J.* **2007**, *408*, 123–130.

(7) Jensen, T. E.; Wojtaszewski, J. F. P.; Richter, E. A. AMP-activated protein kinase in contraction regulation of skeletal muscle metabolism: necessary and/or sufficient? *Acta Physiol.* **2009**, *196*, 155–174.

(8) Shen, Q. W.; Du, M. Role of AMP-activated protein kinase in the glycolysis of postmortem muscle. *J. Sci. Food Agric.* **2005**, *85*, 2401–2406.

(9) Shen, Q. W.; Underwood, K. R.; Means, W. J.; McCormick, R. J.; Du, M. The halothane gene, energy metabolism, adenosine monophosphate-activated protein kinase, and glycolysis in postmortem pig longissimus dorsi muscle. *J. Anim. Sci.* **2007**, *85*, 1054–1061.

(10) Milan, D.; Jeon, J. T.; Looft, C.; Amarger, V.; Robic, A.; Thelander, M.; Rogel-Gaillard, C.; Paul, S.; Iannuccelli, N.; Rask, L.; Ronne, H.; Lundstrom, K.; Reinsch, N.; Gellin, J.; Kalm, E.; Le Roy, P.; Chardon, P.; Andersson, L. A mutation in PRKAG3 associated with excess glycogen content in pig skeletal muscle. *Science* **2000**, *288*, 1248–1251.

(11) Barnes, B. R.; Marklund, S.; Steiler, T. L.; Walter, M.; Hjalm, G.; Amarger, V.; Mahlapuu, M.; Leng, Y.; Johansson, C.; Galuska, D.; Lindgren, K.; Abrink, M.; Stapleton, D.; Zierath, J. R.; Andersson, L. The 5′-AMP-activated protein kinase gamma 3 isoform has a key role in carbohydrate and lipid metabolism in glycolytic skeletal muscle. *J. Biol. Chem.* **2004**, *279*, 38441–38447.

(12) Graves, J. D.; Krebs, E. G. Protein phosphorylation and signal transduction. *Pharmacol. Ther.* **1999**, *82*, 111–121.

(13) Huang, H.; Larsen, M. R.; Karlsson, A. H.; Pomponio, L.; Costa, L. N.; Lametsch, R. Gel-based Phosphoproteomics Analysis of Sarcoplasmic Proteins in Postmortem Porcine Muscle with pH Decline Rate and Time Differences. *Proteomics* **2011**, doi: 10.1002/pmic.201100173.

(14) Lametsch, R.; Kristensen, L.; Larsen, M. R.; Therkildsen, M.; Oksbjerg, N.; Ertbjerg, P. Changes in the muscle proteome after compensatory growth in pigs. *J. Anim. Sci.* **2006**, *84*, 918–924.

(15) Melendez, R.; Melendez-Hevia, E.; Canela, E. I. The fractal structure of glycogen: a clever solution to optimize cell metabolism. *Biophys. J.* **1999**, *77*, 1327–1332.

(16) Hunter, T. Signaling—2000 and beyond. *Cell* **2000**, *100*, 113–127.

(17) Le Roy, P.; Elsen, J. M.; Caritez, J. C.; TALMANT, A.; Juin, H.; Sellier, P.; Monin, G. Comparison between the three porcine RN genotypes for growth, carcass composition and meat quality traits. *Genet., Sel., Evol.* **2000**, *32*, 165–186.

(18) Young, M. E.; Radda, G. K.; Leighton, B. Activation of glycogen phosphorylase and glycogenolysis in rat skeletal muscle by AICAR—An activator of AMP-activated protein kinase. *FEBS Let.* **1996**, *382*, 43–47.

(19) Werner, C.; Natter, R.; Wicke, M. Changes of the activities of glycolytic and oxidative enzymes before and after slaughter in the longissimus muscle of Pietrain and Duroc pigs and a Duroc−Pietrain crossbreed. *J. Anim. Sci.* **2010**, *88*, 4016–4025.

(20) Sola-Penna, M.; Da Silva, D.; Coelho, W. S.; Marinho-Carvalho, M. M.; Zancan, P. Regulation of Mammalian Muscle Type 6-Phosphofructo-1-kinase and Its Implication for the Control of the Metabolism. *IUBMB Life* **2010**, *62*, 791–796.

(21) Schwagele, F.; Haschke, C.; Honikel, K. O.; Krauss, G. Enzymological investigations on the causes for the PSE-syndrome 0.1. Comparative studies on pyruvate kinase from PSE- and normal pig muscles. *Meat Sci.* **1996**, *44*, 27–40.

(22) Takashima, S. Phosphorylation of Myosin Regulatory Light Chain by Myosin Light Chain Kinase, and Muscle Contraction. *Circ. J.* **2009**, *73*, 208–213.

(23) Costa, M. L.; Escaleira, R.; Cataldo, A.; Oliveira, F.; Mermelstein, C. S. Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. *Braz. J. Med. Biol. Res.* **2004**, *37*, 1819–1830.

(24) Flashman, E.; Redwood, C.; Moolman-Smook, J.; Watkins, H. Cardiac myosin binding protein C—its role in physiology and disease. *Circ. Res.* **2004**, *94*, 1279–1289.

(25) Filatov, V. L.; Katrukha, A. G.; Bulargina, T. V.; Gusev, N. B. Troponin: structure, properties, and mechanism of functioning. *Biochemistry (Moscow)* **1999**, *64*, 969–985.

(26) Gordon, A. M.; Homsher, E.; Regnier, M. Regulation of contraction in striated muscle. *Physiol. Rev.* **2000**, *80*, 853–924.

(27) Muroya, S.; Ohnishi-Kameyama, M.; Oe, M.; Nakajima, I.; Shibata, M.; Chikuni, K. Double phosphorylation of the myosin regulatory light chain during rigor mortis of bovine longissimus muscle. *J. Agri. Food Chem.* **2007**, *55*, 3998–4004.