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Chapter 7

INTERRELATIONSHIPS BETWEEN METABOLISM OF GLYCOGEN PHOSPHORYLASE AND PYRIDOXAL PHOSPHATE—IMPLICATIONS IN MCARDLE'S DISEASE

ROBERT J. BEYNON AND CLARE BARTRAM

Department of Biochemistry and Applied Molecular Biology
UMIST
Manchester M60 1QD, United Kingdom

ANGELA FLANNERY, RICHARD P. EVERSHED, AND DEBORAH LEYLAND

Department of Biochemistry
University of Liverpool
Liverpool L69 3BX, United Kingdom

PAMELA HOPKINS

Department of Biochemistry and Applied Molecular Biology
UMIST
Manchester M60 1QD, United Kingdom

VERONICA TOESCU, JOANNE PHOENIX, AND RICHARD H. T. EDWARDS

Muscle Research Centre
Department of Medicine
University of Liverpool
Liverpool L69 3BX, United Kingdom

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I. INTRODUCTION

Glycogen phosphorylase (EC 2.4.1.1., "phosphorylase") is the key regulator of glycogenolysis, catalyzing the phosphorolysis of glycogen to produce glucose-1-phosphate. The enzyme is particularly abundant in muscle, where it functions to provide monosaccharide units for glycolysis in response to demands mediated by endocrine signals or by rises in intracellular calcium.

The cofactor of glycogen phosphorylase is pyridoxal 5′-phosphate (PLP). This cofactor, linked via a Schiff base to a lysine residue (Lys680 in the rabbit sequence), is tightly bound to the enzyme and cannot be resolved from the apo-enzyme unless powerful denaturants are used. The use of PLP in the phosphorylase reaction is unusual and involves the 5′-phosphate group rather than the aldehydic group that is used more commonly in, for example, transaminases (Johnson, 1992).

The control of phosphorylase is subtle, and it is subject to regulation through phosphorylation and through allosteric inhibition (Johnson *et al.*, 1992). It is less clear that changes in intracellular concentration of the enzyme serve a regulatory function, whether mediated through changes in the rates of synthesis or degradation. As part of a study of protein degradation in normal and abnormal skeletal muscle, we have focused on phosphorylase as an abundant sarcoplasmic protein possessing several properties that make it particularly suitable for such studies (Beynon *et al.*, 1993). In this chapter, we review our work on the turnover of glycogen phosphorylase, with particular emphasis on the cofactor as a turnover label.

II. ROLE OF COFACTOR IN PHOSPHORYLASE TURNOVER

Two properties of the muscle phosphorylase pool associated with PLP (its large size and slow kinetics of exchange) led us to speculate that the cofactor might provide a specific label with which to monitor the turnover of the enzyme *in vivo* (Fig. 1). Implicit in this suggestion is the requirement that the cofactor is incorporated into the enzyme as a cosynthetic or immediately postsynthetic event. Further, reutilization of cofactor within mus-

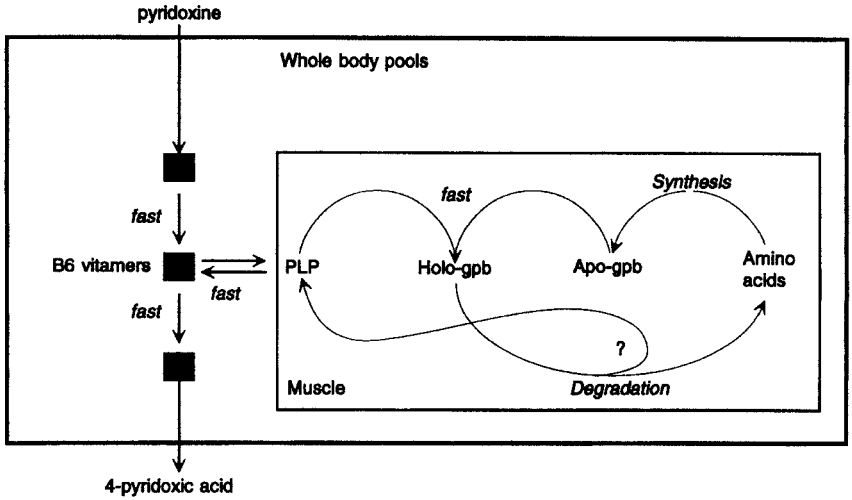

FIG. 1. Interrelationships between vitamin B6 and phosphorylase metabolism. The low rate of turnover of glycogen phosphorylase (gpb) and the lack of exchange of free and protein-bound PLP mean that exchange into the muscle pool is largely controlled by the kinetics of turnover of the enzyme. At present, it is not known whether resolution of the holo-enzyme is a prerequisite or consequence of phosphorylase degradation. Reproduced with permission of The Biochemical Journal.

cle should be minimal, which imposed a requirement for rapid hydrolysis of PLP to pyridoxal (PL) and release of PL from the muscle to the circulation. Our early experiments suggested that these criteria were met. Apo-phosphorylase cannot be detected in muscle, even under conditions of vitamin B6 (pyridoxine, PN) deficiency (R. J. Beynon and D. M. Leyland, unpublished observations). Secondly, low-molecular-weight radiolabeled B6 vitamer pools were cleared very rapidly following a pulse dose of radiolabeled pyridoxine, consistent with a very labile muscle pool of free vitamers. Finally, the rate of degradation of phosphorylase measured by cofactor labeling was the same as that measured by continuous infusion of radiolabeled amino acids (Beynon *et al.*, 1986).

III. LABELING METHODS TO MONITOR PHOSPHORYLASE TURNOVER

A. RADIOLABELED COFACTOR

The principle of using radiolabeled vitamin B6 as a label for phosphorylase degradation is straightforward and takes the form of a classical pulse–

chase protocol (Cookson and Beynon, 1989). Animals were injected subcutaneously with [G-3H] pyridoxine (typically 100–200 μCi for a 25-g mouse). At different times after injection, skeletal muscle was homogenized and a high-speed supernatant fraction (containing virtually all of the radioactivity) was prepared. One portion of the supernatant fraction was separated on G-25 gel filtration to assess the low-molecular-weight pool. A second portion was applied to a column of AMP–Sepharose to bind glycogen phosphorylase, which was subsequently eluted with a solution of AMP. The eluate contained several proteins, but the only PLP-binding protein in the fraction was phosphorylase. This simple, one-step affinity procedure therefore allowed isolation of phosphorylase.

Ten days after injection of label, the low-molecular-weight pool contained virtually no label, whereas label remained associated with phosphorylase. Subsequent isolation of phosphorylase from different animals over a range of time periods between 10 and 30 days defined an exponential decay, the rate constant of which was taken as the rate of degradation of phosphorylase. This was subsequently confirmed by independent measurement of the rate of turnover of the enzyme using continuous infusion of labeled amino acids—a method that is independent of reutilization artifacts. The rate of turnover of phosphorylase was the same when measured by either method (Beynon *et al.*, 1986; Cookson and Beynon, 1989).

B. STABLE ISOTOPE-LABELED COFACTOR

Phosphorylase has a relatively low rate of turnover, and as such, large doses of radiolabeled pyridoxine were needed to obtain an adequate degree of labeling. In addition, the need for serial sampling of the decay curve, using individual animals, introduced substantial biological variation. Both aspects of this experimental system precluded application of the method to humans, and we considered the possibility of a different approach, based on stable-isotope-labeled pyridoxine, to monitor phosphorylase degradation.

It has long been recognized that the simplest model of vitamin B6 metabolism required two pools, a small, mobile pool and a large, slow pool. Coburn and colleagues (reviewed in Coburn, 1990) have proposed that the only source of the large, slow pool is muscle glycogen phosphorylase—large because of the abundance of phosphorylase in skeletal muscle and slow because of the low rate of exchange of label into and out of this protein. This suggestion was based on calculations of the accessibility of this pool (Coburn *et al.*, 1991) and from muscle biopsy studies (Coburn *et al.*, 1988). We believe that the rate of exchange into and out of this pool reflects the rate of turnover of the enzyme.

Dideuterated pyridoxine was synthesized according to methods developed by Coburn *et al.* (1982) and was purified and administered to mice in drinking water. The animals consumed a vitamin B6-deficient diet for the duration of the experiment. At times throughout the experiment, urine samples were taken and the excreted 4-pyridoxic acid was analyzed as the *tert*-butyldimethylsilyl derivative by GC/MS in selected ion monitoring mode. The *tert*-butyl group is lost to yield a fragment ion of *m/z* 224 (*m/z* 222 for unlabeled samples) corresponding to the monoderivatized lactone of 4-pyridoxic acid (Leyland *et al.*, 1992).

The technique of oral administration of dideuterated PN and analysis of urinary output of labeled 4-pyridoxic acid has the advantage of being noninvasive, and, of course, repeated urinalysis permits definition of the labeling curve in a single animal over an extended period. We have analyzed the labeling kinetics of mice over a 50-day period using this technique (Fig. 2). At the end of this period the isotope abundance has almost attained that of the ingested material, although the failure to reach maximal isotope abundance may reflect a low level of pyridoxine in the diet formulation.

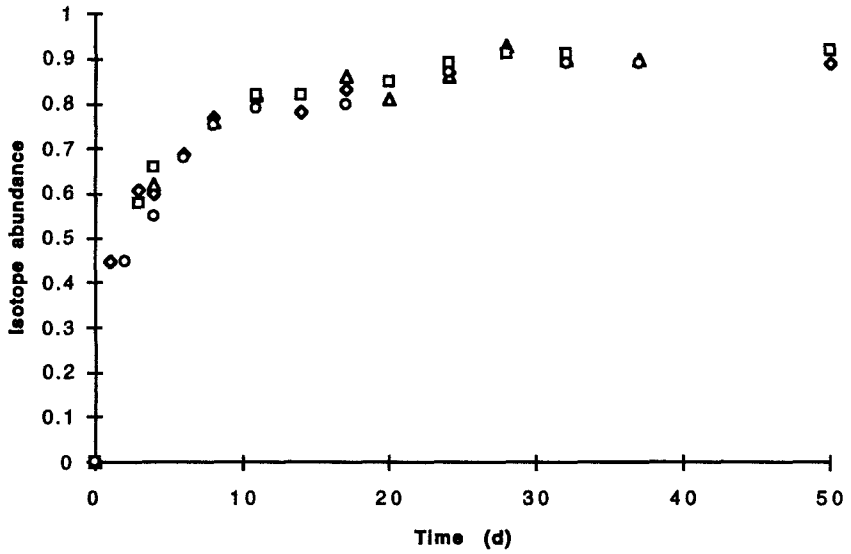

FIG. 2. Whole animal labeling kinetics with stable-isotope-labeled pyridoxine. Mice were placed on a vitamin B6-deficient diet and labeled with dideuterated pyridoxine. At intervals, urine samples were collected and the isotope abundance of excreted 4-pyridoxic acid was measured. After 50 days, the animals have attained a plateau labeling. When analyzed as a biexponential process, the rate constant for the labeling of the slow pool is the same as that obtained by pulse labeling with radioactive pyridoxine. The symbols correspond to the data from four individuals in the study.

The kinetics of the rise to a plateau value can be analyzed by nonlinear curve fitting as a biexponential equation and yields rate constants for turnover of the large and slow components, from which the rate constant of loss of PLP from the phosphorylase pool (=rate of degradation of enzyme) can be calculated (Coburn, Chapter 6, this volume).

The rate constant for turnover of the slow component was 0.13 ± 0.03/day (mean ± SEM., $n = 10$) from which a value of turnover of the phosphorylase pool can be calculated as 0.1/day (Beynon *et al.*, 1996). This compares well with values of 0.12/day obtained for gastrocnemius muscle (Leyland *et al.*, 1990) and 0.13/day for total hind limb and back muscle (Leyland and Beynon, 1991). The fast pool (presumed to be all labile forms of the vitamin) was turning over very quickly, with a rate constant of 1.3 ± 0.4/day (a half-life of 12 hr). However, the experimental protocol that we use does not permit acquisition of a sufficiently detailed data set to acquire accurate kinetics on the fast pool. This preliminary analysis of the data also implies that the fast pool accounts for about 50% of the total vitamin B6 in the body—it is not yet clear whether this is consequential to the inability to define the fast phase with a high degree of precision or whether the muscle phosphorylase itself partitions into two pools that differ in accessibility. For example, enzyme bound to the glycogen particle might be more stable than enzyme free in the sarcoplasm. Further work is needed to resolve these issues.

IV. MODEL SYSTEMS FOR PHOSPHORYLASE EXPRESSION

Muscle wasting or growth is a result of imbalance between the opposing processes of protein synthesis and degradation, and, in many conditions, the relative contributions of the two are not known. Moreover, many studies on protein turnover analyze total protein pools, which obscures the behavior of individual proteins within those pools. The pyridoxine labeling methods that we have developed have allowed us to explore phosphorylase degradation under a number of different conditions. Two models of muscle wasting processes have been studied in the mouse, and the role of degradation in muscle growth has focused on the chicken.

A. MUSCLE WASTING

Two models for muscle wasting have been studied in the mouse (Table I). Section of the sciatic nerve causes a rapid denervation-induced atrophy in the lower limb muscles, and we have measured different parameters of phosphorylase expression in the gastrocnemius of the mouse after unilateral

TABLE I

EXPRESSION OF PHOSPHORYLASE IN TWO MOUSE MODELS FOR MUSCLE WASTING

Animal model	k_d[a]	Phosphorylase (SA[b], % control)	mRNA (% of control)
C57BL/6J denervation			
Control limb	0.12/day	—	—
Operated limb	0.2/day	30	50
C57BL/6Jdy/dy			
Control	0.13/day	—	—
Mutant	0.05/day	40	60

[a] Rate constant for degradation.
[b] Specific activity.

denervation, using the contralateral muscle as a control (Leyland *et al.*, 1990). The response to denervation is biphasic. In the first 4–5 days after denervation, phosphorylase mRNA levels decline markedly, to about 50% of the values in the control limb. Phosphorylase content in muscle (measured by activity) declines slowly over this first phase but in the second period (5–25 days) disappears from the muscle more quickly. The specific activity of the enzyme declines during the experimental period to 30% of control values, indicating preferential loss of this enzyme relative to other muscle proteins. The acceleration in the loss of enzyme is reflected in enhanced degradation of 0.2/day compared to 0.11/day in the control limb. Although we have not measured rates of synthesis directly, it is not unreasonable to assume that the lower mRNA abundance is manifest as a lower rate of synthesis. Thus, loss of phosphorylase is accomplished by a lower rate of synthesis and an accelerated rate of degradation. Further evidence for enhanced degradation comes from Western blot studies with a monoclonal antibody to the PLP—a transient enhancement of degradation intermediates is apparent (Cookson *et al.*, 1992).

Completely different behavior is seen in the C57BL/6Jdy/dy dystrophic mouse (Leyland and Beynon, 1991). This is a severe muscle wasting condition, unrelated to the less severe C57BL/10J$^{mdx/mdx}$ mutant that is dystrophin deficient. The mutation in the C57BL/6J$^{dy/dy}$ animal is not known. In dystrophic animals, the rate of degradation of phosphorylase is less than in normal animals (0.05/day, compared to 0.13/day) and the mRNA level is 60% of normal values. By implication, the rate of phosphorylase synthesis in these animals is severely suppressed, consistent with an overall metabolic downregulation in this condition. The failure to detect degradation intermediates such as those seen in denervation atrophy adds further credence to this view.

B. MUSCLE GROWTH

For analysis of phosphorylase expression in muscle growth, we have focused on the chicken. In particular, we have compared animals selected for rapid growth (broiler) with animals selected for egg production (layer). In this study, we concentrated on the pectoralis muscle. During the period of the experiment, from 2 to 8 week, the pectoralis muscle increases in mass from 15 to 40 grams in the layer and from 13 to 160 grams in the broiler. The total pool of phosphorylase expands by 3-fold in the layer compared to 19-fold in the broiler. How is this dramatic expansion of the phosphorylase pool achieved in the broiler—by enhanced synthesis or by suppressed degradation? In this system, it was necessary to modify the kinetic analyses to address the issue of: (a) an expanding tissue pool and (b) the potential for a time-dependent change in turnover rate throughout the growth period (Flannery *et al.*, 1992).

The results of our analyses are summarized in Fig. 3. In 3-week-old animals, when the pectoralis weight is the same in broilers and layers, the rate of phosphorylase synthesis is similar in the two strains, at approx 0.1/day. The strains differ markedly in the rate of degradation of the enzyme: broiler, approx 0.01/day, and layer, approx 0.05/day. As the animals grow, the two strains yield very different results. At 8 weeks in the broiler, the synthesis rate declines slightly, to 0.07/day whereas in the layer, it declines to 0.04/day. Thus, the synthesis of the enzyme is not very different in the two strains. By contrast, the rate of degradation of the enzyme declines to 0.005/day in layer, but remains at 0.01/day in the broiler.

Thus, several factors combine to produce the different growth behaviors. In layers, the age-dependent decline in the rate of turnover of the protein and the convergence of the rates of synthesis and degradation are similar to the growth pattern seen in many species and tissues.

In the broilers, rapid growth is achieved by a combination of two factors. First, even in young animals, the rate of degradation of the enzyme is low. Second, synthesis and degradation rates do not converge as quickly in the broilers, allowing for a rapid growth that soon outstrips that of the layer. It seems likely that these animals have been selected for a sustained downregulation of the degradative machinery or for an early maturation-dependent decline in this machinery. Analysis of the expression of these systems in even younger animals might prove fruitful.

V. VITAMIN B6 AND MCARDLE'S DISEASE

Our interest in phosphorylase turnover has led us to appreciate the importance of the phosphorylase-bound pool of vitamin B6. The behavior

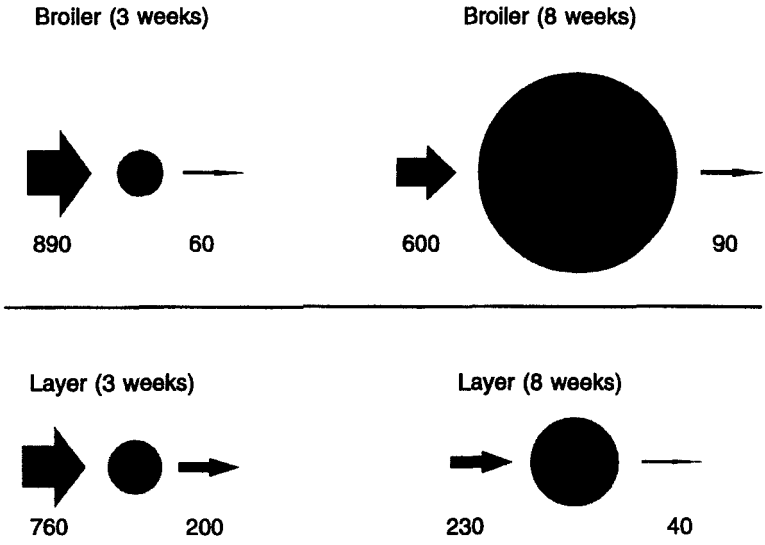

FIG. 3. Expression of glycogen phosphorylase in growing chicken pectoralis muscle. The total phosphorylase pool sizes are represented by the areas of the circles and are given here in milligram total followed by the concentrations (mg/g) in parentheses: broiler 3 week, 75 (5.2 ± 0.4); broiler 8 week, 1410 (8.2 ± 0.2); layer 3 week, 100 (5.1 ± 0.5); layer 8 week, 270 (6.6 ± 0.8). The fluxes into and out of the phosphorylase pool are presented per gram of tissue and are given in μg/day/gram wet weight tissue—this is more representative of the metabolic activity per mass of tissue, in a rapidly expanding muscle. The width of the arrows into and out of the pools are in proportion to their magnitude. The convergence of the synthesis and degradation rates in layers is apparent as is the sustained imbalance between the two processes in broilers. Unlike layers, the specific activity of the enzyme in the 8-week broiler pool is about 50% higher than the starting material, represented by a stronger shading.

of this pool has consequences for recommendations of vitamin B6 intakes (Coburn, 1990), and the inaccessibility of the pool may stress the different requirements for vitamin B6 during growth, when the phosphorylase pool is accreting, and in the adult, where vitamin B6 requirements may function primarily to sustain throughput of the fast pools. In principle, PLP could be released into the body at a maximal rate equal to that of phosphorylase degradation, but it is unlikely that phosphorylase acts as a "store" of vitamin B6 in the body.

If the phosphorylase pool plays an important part in vitamin B6 kinetics, it might be anticipated that this metabolism would be disturbed in patients suffering from McArdle's disease, a rare metabolic myopathy caused by an absence of functional muscle glycogen phosphorylase. The absence of this enzyme means that patients cannot break down their muscle glycogen reserves. Other energy sources within the muscle are rapidly depleted

during exercise but cannot be replenished quickly enough to allow normal muscle action. As a consequence, the clinical symptoms of the disease include muscle pain and cramps induced by exercise.

A. BIOCHEMICAL HETEROGENEITY

There are a number of steps in the expression of a functional protein from a gene and a disruption at any point can halt the normal expression of the protein. Consequently, there is variation between patients in their expression of phosphorylase mRNA and protein (McConchie *et al.,* 1991). The majority of individuals do not express protein or mRNA, which is due either to a failure to transcribe the gene or due to accelerated degradation of the transcript. A smaller number of patients do express a stable transcript but it is not subsequently translated into protein and even fewer patients express protein but at low levels (approximately 1% of normal values). The majority of McArdle's patients are characterized by a complete absence of the protein in muscle, consistent with knowledge of the mutations in McArdle's disease.

B. MOLECULAR GENETICS

McArdle's disease is a recessive condition and one normal copy of the gene seems to be adequate for normal phosphorylase activity in the muscle. In common with most molecular diseases, it is characterized by a number of different mutations (Fig. 4). The most frequently occurring of these is a single base change which generates a premature stop codon and has been

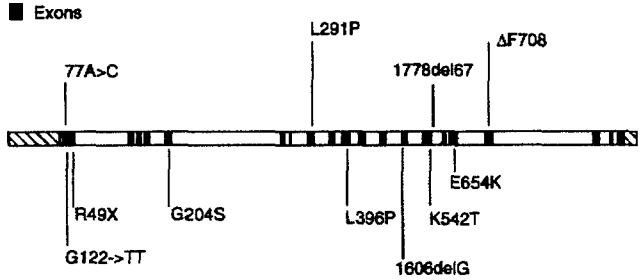

FIG. 4. Mutations in the myophosphorylase gene in McArdle's disease. The mutations are described in the text. The most common phenotype is a complete lack of phosphorylase protein in skeletal muscle and, hence, the loss of the muscle vitamin B6 "slow" pool.

designated the R49X mutation (Bartram *et al.*, 1993; Tsujino *et al.*, 1993). This mutation generates a truncated peptide that is likely to be rapidly degraded but it also causes instability of the transcript—patients homozygous for the R49X mutation have no detectable mRNA (Bartram *et al.*, 1993, 1995). A rare frameshift mutation, designated the 122G → TT mutation, also causes premature termination of the protein (Bartram *et al.*, 1994). Three missense mutations implicated in the disease, L291P, G204S, and K542T, do not prevent translation of the protein but are postulated to have an effect on the normal function of the enzyme or its stability (Tsujino *et al.*, 1993, 1994). The L291P mutation may have a structural effect upon the protein whereas the G204S mutation is associated with a glycogen-binding domain and K542T affects a glucose-binding domain. In addition to the above, a mutation which deletes a single codon, ΔF708, may have a structural effect. Finally, a splice junction mutation at the boundary between exon 14 and intron 14 results in a 67-bp deletion in the transcript due to a frameshift that causes premature termination of the protein (Tsujino *et al.*, 1994). These seven mutations do not explain all cases of McArdle's disease—there are still other unknown mutations (Bartram *et al.*, 1995).

C. MCARDLE'S DISEASE AND VITAMIN B6 METABOLISM

Our interest in McArdle's disease and vitamin B6 metabolism is stimulated by consideration of the consequences of the loss of the major, slowly metabolizing pool of vitamin B6 in the body. It is conceivable that the whole body phosphorylase-derived pool acts as a "buffer" to compensate for day-to-day variation in vitamin B6 intake. It will therefore be important to assess the rate of degradation of phosphorylase in the human, and the stable isotope method we have developed is directly applicable to this problem. Analysis of the same kinetics in McArdle's patients will define the role of muscle phosphorylase in the compartmentalization of vitamin B6.

It is conceivable that McArdle's patients need to pay greater attention to their vitamin B6 status than normal individuals. A compromised vitamin B6 status might be of even greater significance if McArdle's patients are more reliant on amino acid metabolism for muscle work—transaminases are PLP-dependent enzymes. Preliminary studies in our laboratory have established that McArdle's patients show differences in vitamin B6 metabolism and that they respond quickly and dramatically to short-term changes in vitamin B6 status (Beynon *et al.*, 1995). Whether improvement of vitamin B6 status could enhance muscle performance remains to be seen.

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