SKELETAL MUSCLE PHOSPHORYLASE KINASE DEFICIENCY: DETECTION OF A PROTEIN LACKING ANY ACTIVITY IN ICR/IAn MICE

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1. Introduction

The total absence of phosphorylase kinase in skeletal muscle of I strain mice was originally described by Lyon and coworkers [1–3]. This finding was of considerable interest in view of the extremely mild symptoms accompanying the deficiency, and the central importance of phosphorylase kinase in linking the nervous and hormonal control of glycogen mobilisation in muscle [4]. In addition, the mutant mice were reported to breakdown glycogen in response to both electrical stimulation and adrenalin [1, 2], observations difficult to reconcile with the absence of this enzyme. The problem appeared to be resolved rather simply, when Huijing reported that phosphorylase kinase was not completely absent in I strain mice, but was present in a particulate fraction removed with the structural muscle proteins during the initial centrifugation of the homogenised muscle extract [5].

In this communication we show that the activity observed in the particulate fraction is not phosphorylase kinase, but due to the production of AMP with the subsequent activation of phosphorylase *b*. Furthermore immunological studies demonstrate that an inactive phosphorylase kinase protein is present in the supernatant fraction of muscle homogenates prepared from phosphorylase kinase deficient mice.

2. Materials and methods

2.1. Mice

A colony of ICR/IAn mice, carrying the X-linked gene for phosphorylase kinase deficiency was established from three breeding pairs, kindly provided by Dr. Muriel N. Nesbitt, Department of Psychiatry, University of California, Los Angeles, USA. These animals originated from the random bred strain ICR, to which the X-chromosome of the inbred strain I/An (carrying the phosphorylase kinase deficient gene) was transferred by cross-breeding for 20 generations. The strain has subsequently been maintained by breeding within itself.

A colony of C3H/He/mg mice was bred from four mating pairs, obtained from the Medical Research Council Laboratory at Carlshalton, Surrey, England. These animals were used as controls possessing "normal" enzyme activity.

2.2. Muscle extracts

Mice were killed with ether and skeletal muscle from the limbs and back was excised and chilled in ice. The muscle was chopped finely with scissors, and homogenised in a Vertis 23 homogeniser with 50 ml of ice-cold water per gram of tissue. Assays were carried out either directly with this homogenate or with a supernatant obtained by centrifugation at 10,000 *g* for 20 min.

2.2. Assays of phosphorylase kinase

The routine assay of Krebs [6] was modified as follows: 0.125 M Tris–0.125 M glycerophosphate pH 8.6 (adjusted to pH with HCl) 0.05 ml; rabbit muscle phosphorylase *b* 15 mg/ml 0.05 ml; calcium chloride 3 mM 0.01 ml; homogenate or supernatant 0.02 ml; 75 mM $MgCl_2$ –22.5 mM ATP pH 7.0, 0.02 ml. The last component initiated the reaction. After 5 or 30 minutes

at 30° an aliquot was diluted 40-fold in 0.1 M sodium maleate–0.1% bovine serum albumin–50 mM 2-mercaptoethanol pH 6.5 at 0° and assayed for phosphorylase *a* in the direction of glycogen synthesis [7]. One unit of phosphorylase kinase is defined as the number of micromoles of phosphorylase *b* converted to phosphorylase *a* per minute. For this calculation, the absorbance index of phosphorylase, $A_{280}^{1\%}$, was taken as 13.1, the monomer molecular weight as 100,000 and the specific activity of phosphorylase *a* as 54 units per mg [8].

Rabbit muscle phosphorylase, prepared by the method of Fisher and Krebs [9] was recrystallised three times. The crystals were redissolved in 50 mM glycerophosphate–2 mM EDTA–50 mM 2-mercaptoethanol buffer pH 7.0 and AMP removed by passage through charcoal. The AMP free enzyme possessed a 260/280 nm absorbance ratio of 0.53–0.54.

2.4. Rabbit muscle phosphorylase kinase

Was prepared by a modification of the procedure of Brostrom et al. [10] and was homogeneous by the criteria of sedimentation velocity and DEAE-cellulose chromatography (Philip Cohen, in preparation).

2.5. Preparation of antiserum to phosphorylase kinase

Purified rabbit muscle phosphorylase kinase (0.8 ml) containing 4 mg of enzyme in 50 mM glycerophosphate–2 mM EDTA–0.15 M NaCl pH 6.8 was mixed with an equal volume of Freund's complete adjuvant to form a fine emulsion. The material was injected subcutaneously into each of two hens and a third control animal was injected with Freund's adjuvant alone. The procedure was repeated after 2 weeks. Blood was withdrawn 6 days after the second injection allowed to clot, and the serum collected by centrifugation. Antigen–antibody precipitation was carried out by Ouchterlony double diffusion in 1% agar buffered with 50 mM glycerophosphate–2 mM EDTA pH 6.8 containing 1.5 M NaCl [11].

Table 1
Phosphorylase kinase in mouse skeletal muscle extracts.

Source of enzyme	Phosphorylase kinase activity (units/g tissue)	Number of mice tested
C3H/He/mg supernatant or homogenate	0.7 ± 0.05	6
ICR/IAn supernatant	0	10
ICR/IAn homogenate	0.028	3

3. Results and discussion

3.1. Activity of phosphorylase kinase in the skeletal muscle homogenate and 10,000 g supernatant

While phosphorylase kinase was present in the 10,000 *g* muscle supernatant of normal C3H/He/mg mice, no activity was detectable in the muscle supernatant from the phosphorylase kinase deficient ICR/IAn strain, confirming previous results [1–3]. However, in the homogenate of the ICR/IAn strain, an activity 4–5% of normal was observed, again as described previously [5]. The results are given in table 1.

Although the latter observation was interpreted as suggesting that phosphorylase kinase in the mutant mice was particulate [5], no control experiments were performed to eliminate the possibility that this activity might be due to AMP production during the assay with subsequent activation of phosphorylase *b*. Indeed, a homogenate would be expected to possess the powerful ATPase activity associated with the structural muscle proteins, which, in conjugation with adenylate kinase, would rapidly produce AMP. The following experiments were therefore designed to ascertain the nature of the "phosphorylase kinase activity" observed in the ICR/IAn homogenate.

The phosphorylase *b* concentration was varied in the phosphorylase kinase assay. With the muscle supernatant of C3H/He/mg mice, phosphorylase kinase activity increased with increasing phosphorylase *b* concentration over the range 1–10 mg/ml (fig. 1). In contrast, the "phosphorylase kinase activity" measured in the ICR/IAn muscle homogenate showed no such dependence on phosphorylase *b* concentration. Indeed, when phosphorylase kinase was assayed at different phosphorylase *b* concentration, and aliquots diluted appropriately to maintain a constant total phosphorylase concentration and then assayed for the phosphorylase *a* formed, results were obtained as shown in fig. 2. An apparent increase in "activity" occurred with de-

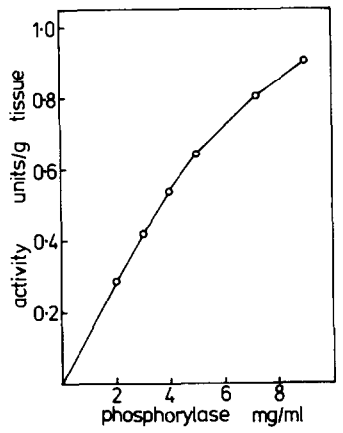

Fig. 1. Phosphorylase kinase activity in the muscle supernatant of C3H/He/mg mice assayed at varying phosphorylase *b* concentrations.

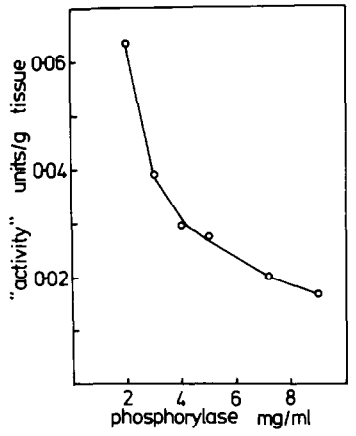

Fig. 2. "Phosphorylase kinase activity" in the muscle homogenate of ICR/IAn mice assayed at varying phosphorylase *b* concentrations. The experiment was carried out as described under Results.

creasing initial phosphorylase *b* concentration immediately suggesting that AMP was being produced. Thus low dilution of the aliquot at low phosphorylase concentration carried over more AMP to the assay for phosphorylase *a* formed.

Further confirmation that activity in the ICR/IAn *homogenate* did not involve conversion of phosphorylase *b* to *a* was obtained by omitting the phosphorylase *b* from the initial kinase assay, but then including it in the dilution buffer for the phosphorylase *a* measurement. This gave the same activity for the ICR/IAn *homogenate* as in the standard assay, demonstrating formation of a compound (presumably AMP or IMP) capable of activating phosphorylase *b*. This compound was also stable to boiling. In contrast *supernatants* of both C3H/He/mg and ICR/IAn assayed in the same manner gave zero activity.

Table 2
Nucleotide levels after phosphorylase kinase assays.

Extract used in assay	ATP (mM)[a]	ADP (mM)[b]	AMP (mM)[b]
C3H/He/mg supernatant	2.84	0.14	0.03
ICR/IAn supernatant	2.78	0.24	0.03
ICR/IAn homogenate	1.45	1.04	0.62

[a] Concentration at the end of the assay. The initial concentration was 3.07 mM.
[b] Formed during the assay.

The C3H/He/mg supernatant was assayed for 5 min and the ICR/IAn supernatant and homogenate for 30 min in the standard assay described under Methods.

The above results were substantiated by direct measurement of the ATP [12], ADP and AMP [13] levels in kinase assays of both supernatants and homogenate. The results are given in table 2. The supernatants from C3H/He/mg and ICR/IAn mice form only 0.03 mM AMP in the assays, while 0.62 mM AMP is generated by the ICR/IAn homogenate. (A similar amount of AMP was also produced by the C3H/He/me homogenate.) Since the kinase incubation is diluted 40-fold before assaying for formation of phosphorylase *a*, the concentration of AMP is 0.75×10^{-6} M with the supernatants, but 16×10^{-6} M with the ICR/IAn homogenate. From the K_m of activation of AMP for phosphorylase *b* in this system, which is 1.4×10^{-4} [9], it may be calculated that 0.62 mM formed in the above assay is almost exactly right to account for the 5% of normal "activity" observed in the standard assay with the ICR/IAn homogenate. We therefore conclude that extracts of ICR/IAn muscle do not contain a particulate phosphorylase kinase.

3.2. Detection of a mutant phosphorylase kinase protein

Homogenisation of muscle in 2.5 vol of 4 mM EDTA pH 7.0, rather than 50 vol of water, to increase the sen-

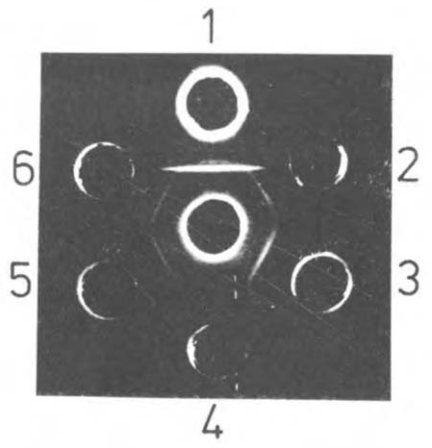

Fig. 3. Ouchterlony double diffusion in agar. Centre well: Antiserum to purified rabbit muscle phosphorylase kinase, 40 μl. Outside wells: 1) Rabbit muscle phosphorylase kinase 5 mg/ml 40 μl. 2) and 5) Supernatant from C3H/He/mg mice 40 μl. 3) and 6) Supernatant from ICR/IAn mice 40 μl. 4) Rabbit muscle phosphorylase 5 mg/ml 40 μl.

sitivity of the assay 20-fold, failed to reveal any phosphorylase kinase activity in the 10,000 *g* supernatant of the ICR/IAn strain. Thus if any activity is present it must be less than 0.2% of the normal value under the standard assay conditions. Absence of activity in the muscle cell is indicated by inability to demonstrate any phosphorylase *a* formation *in vivo* [1, 2].

Immunological studies were carried out to determine whether an inactive phosphorylase kinase was being synthesized in the ICR/IAn mice. The results of Ouchterlony double diffusion experiments with antiserum to rabbit muscle phosphorylase kinase are shown in fig. 3. Antiserum yielded a single precipitin line with purified rabbit phosphorylase kinase. Both ICR/IAn and C3H/He/mg supernatants also showed cross reacting material. A control hen injected with Freund's adjuvant gave no precipitin line (not illustrated). The precipitin lines formed by ICR/IAn and C3H/He/mg were of similar intensity suggesting the cross reacting material was in similar concentration in both supernatants. No spur formation could be detected between the two mouse precipitin lines, although each gave spur formation with that formed with rabbit muscle phosphorylase kinase.

These studies indicate that although the ICR/IAn mice have essentially zero phosphorylase kinase activity, they contain a mutant protein which is present in similar concentrations to the normal enzyme, and immunologically indistinguishable from it by double diffusion criteria. This inactive phosphorylase kinase is currently being purified and characterized. Investigation of its structure should allow elucidation of the molecular basis of the lesion, which is of additional interest, since the deficiency shows X-chromosomal inheritance [3] and mammalian phosphorylase kinases are now known to be composed of three types of polypeptide chain [14].

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