Presence of NAD pyrophosphorylase in skeletal muscle in dystrophic mice

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Summary. NAD pyrophosphorylase (ATP:NMN adenylyltransferase) activity has been measured in the skeletal muscle of dystrophic mice. The amount of this enzyme in the dystrophic mice, as determined by three different methods, was about one half of that in the controls. In addition, the concentration of ATP was too low to be detected in crude extracts of dystrophic mouse skeletal muscle, which were prepared using Tris buffer alone or Tris buffer containing either 3 M KCl, or 1 mM PMSF.
Key words. NAD metabolism; nucleotide metabolism; Duchenne dystrophy.

The metabolic disorder which appears to develop in dystrophic muscle, parallel to the development of the disease, suggests an involvement of nucleotide metabolism and the enzymes involved in it.

In particular, the intracellular NAD levels seem to play an important role in the control of the muscular differentiation process[1-3]. The total NAD level of dystrophic muscle is low compared with that in normal muscle. Low levels of NAD are associated in in vivo experiments with chondrogenic cells, and high levels of NAD are associated with myogenic cells[3].

The importance of nuclear NAD levels is related to nuclear events during cellular life (differentiation, division, DNA repair). In particular, nuclear NAD is the substrate of two chromatin-associated enzymes. The first one, poly(ADP-ribose)polymerase, converts NAD into poly(ADP-ribose) with the excision of nicotinamide.

The second one, ATP:NMN adenylyltransferase, converts NAD and pyrophosphate into NMN and ATP, and vice-versa.

We have focused our attention on ATP: NMN adenylyltransferase, which we formerly purified and characterized from bakers' yeast[4].

Materials and methods

NMN, NAD and ATP were obtained from Sigma. (8-^{14}C)ATP was from Amersham. All other chemicals were of analytical-reagent grade or HPLC grade. Cellulose thin layer sheets with fluorescent indicator were from Eastman Kodak. Enzymes and protein markers were from Sigma or Boehringer Mannheim. LC-18, 5 µm, 25 cm HPLC column was from Supelco. Dystrophic male mice (6–7 weeks old) of the C57BL/6J-dy/dy strain and controls of the same age, C57BL/6J-dy/+, were purchased from the Jackson Laboratory.

Preparation of muscle extracts. The mice were killed by cervical dislocation and the hind leg muscles were removed immediately and immersed in liquid nitrogen. The tissue samples were pulverized in a precooled mortar and after the addition of 10 ml/g tissue of either 50 mM Tris-HCl pH 7.6 or 50 mM Tris-HCl pH 7.6 containing 3 M KCl, or 50 mM Tris-HCl pH 7.6 containing 1 mM PMSF, were homogenized in an Ultra-Turrax F25 homogenizer (Janke & Kunkel) 24000 rpm, 5 s, and centrifuged at 48000 × g for 20 min. The supernatants were the crude extracts which were used for the following experiments.

Determination of nucleotide concentrations. The extraction of nucleosides and nucleotides from the crude extracts, and their determination using HPLC, were performed as described by Beutler et al.[5], as reported by Stocchi et al.[6] with minor modifications. To a 100-µl sample, 50 µl of 1.2 M $HClO_4$ was added. After centrifugation, to 130 µl of supernatant 30 µl of 1 M K_2CO_3 was added, bringing the pH to 6.0. After removal of insoluble $KCLO_4$ by centrifugation, 50 µl of supernatant was injected onto a Supelco LC-18, 5 µm, reverse phase column (4.6 × 250 mm). The column was previously standardized by using 2–10 nmol of a standard mixture of the appropriate nucleosides and nucleotides. The elution was performed with the following gradient: 9 min at 100% buffer A; 6 min at 12.5% buffer B; 2.5 min at 45% B; 2 min at 100% B; after 6 min at 100% B, the gradient was returned to 100% A in 5 min. Buffer A was 0.1 M potassium phosphate buffer, pH 6; buffer B was buffer A containing 20% methanol. The flow rate was 1.3 ml/min and detection was at 254 nm.

Assay of enzyme activity. Enzyme activity was routinely tested by a continuous spectrophotometric coupled enzyme assay modified after Kornberg[7]. The reaction mixture contained, in a final volume of 0.85 ml, 60 mM HEPES buffer, pH 7.6, NMN 1.18 mM, ATP 1.47 mM, $MgCl_2$ 20.7 mM, semicarbazide-HCl 35 mM, ethanol 0.45%, yeast alcohol dehydrogenase, 7.8 U, bovine serum albumin 0.59 mg/ml and the appropriate amount of sample to be assayed. The reaction was started by addition of NMN and continuously followed at 340 nm in a Varian DMS 90 spectrophotometer. The temperature was maintained at 37 °C.

Alternatively, the activity was assayed by measuring the radioactivity incorporated into NAD from labeled ATP, after electrophoresis on a cellulose thin layer (20 × 20 cm)

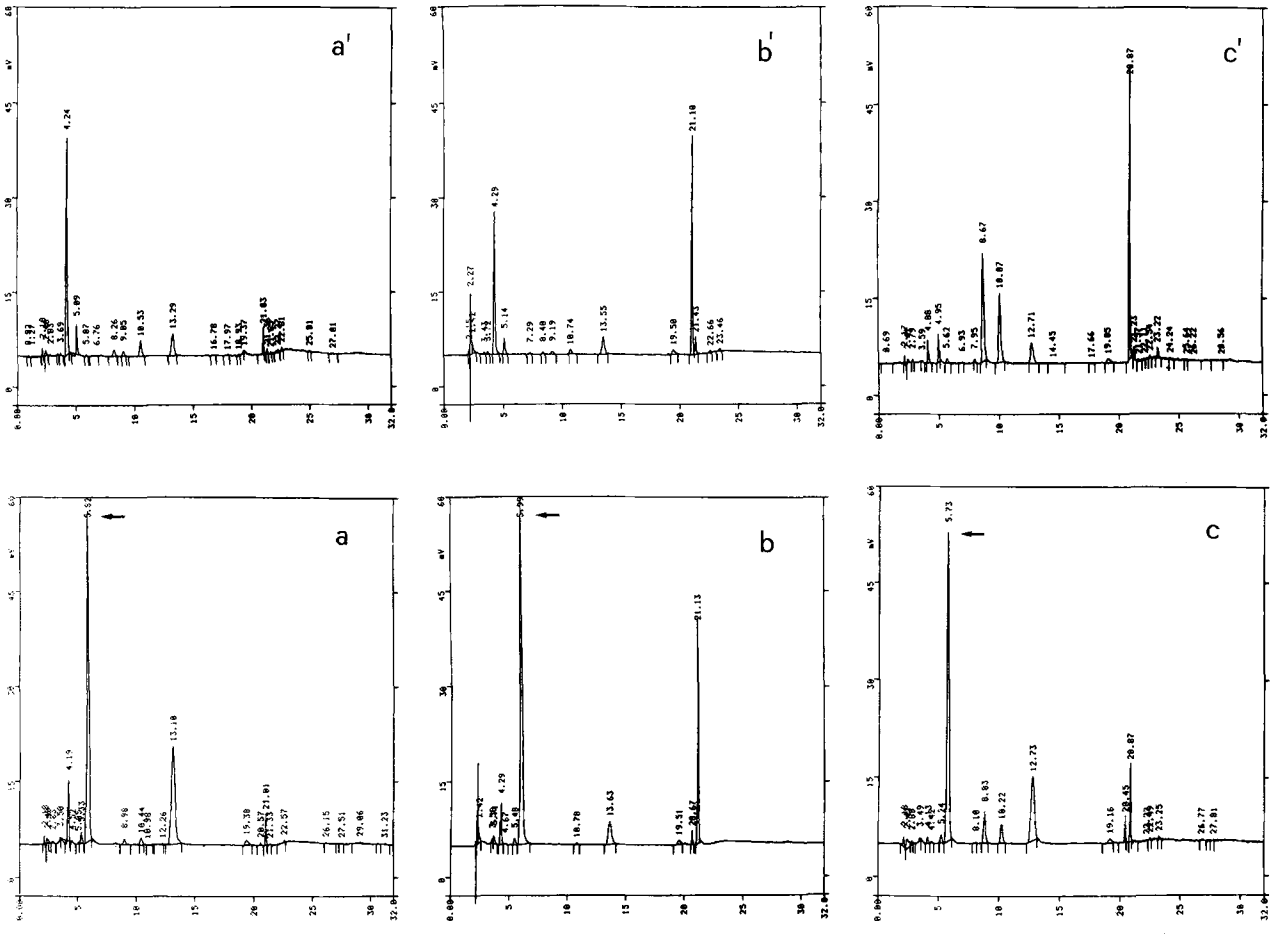
ATP: NMN adenylyltransferase activity and ATP content in the crude extracts

Extraction buffer	Proteins (mg/ml)		Enzyme activity (U*/ml)		Specific activity (U/mg)		Units/g (wet tissue)		ATP, µmols/g (wet tissue)	
	c°	d^	c	d	c	d	c	d	c	d
Tris-HCl 50 mM pH 7.6	5.0	5.3	0.012	0.006	0.0024	0.001	0.12	0.06	3.46	§n.d.
Tris-HCl 50 mM pH 7.6 containing 3 M KCl	10.0	9.0	0.025	0.013	0.0025	0.001	0.25	0.13	3.84	n.d.
Tris-HCl 50 mM pH 7.6 containing 1 mM PMSF	5.2	4.9	0.012	0.0058	0.0023	0.001	0.12	0.058	3.3	n.d.

*One unit of enzyme activity produces 1 µmol of NAD per min. °c = controls. ^d = dystrophic mice. §n.d. = not detectable.

to separate the above-mentioned nucleotides, using 50 mM citrate buffer pH 5.0, at 400 V for 3.5 h. The reaction mixture in this case contained, in a final volume of 100 µl, 2.25 mM (8-^{14}C)ATP (5.0 mCi/nmol), 2.5 mM NMN, 60 mM HEPES pH 7.6, 20 mM $MgCl_2$ and the appropriate amount of enzyme. After 30 min incubation at 37 °C the reaction was stopped by addition of 10 µl 40% (w/v) trichloroacetic acid. After centrifugation, 10 µl of supernatant was spotted on a cellulose thin layer together with appropriate amounts of cold NAD and ATP. After electrophoresis and drying, the spots corresponding to NAD and ATP were cut out, placed in vials with 12 ml scintillation mixture and counted in a Phillips PW4540 liquid scintillation spectrometer.

Alternatively the NAD and ATP concentrations were determined on an HPLC LC-18 column using the conditions reported above for nucleoside and nucleotide determination.



HPLC reverse phase on a 5-µm (4.6 × 250 mm) Supelcosil C-18 column. The column was calibrated using 2–10 nmol of nucleotides and nucleosides and eluted as described under 'Materials and methods'. As shown, the ATP nucleotide is present only in the crude muscle extract of controls (a,b,c) and not in those of dystrophic mice (a',b',c'). When the extraction was conducted in the controls, by Tris-HCl 50 mM pH 7.6 (a), by Tris-HCl 50 mM pH 7.6 containing 3 M KCl (b), or by Tris-HCl 50 mM pH 7.6 containing 1 mM PMSF (c), we found 9 nmoles, 10 nmoles and 7.9 nmoles of ATP respectively, corresponding to 3.46 µmoles, 3,84 µmoles and 3.3 µmoles per g of wet tissue. No detectable amount of ATP was found in the samples deriving from dystrophic mice (a',b',c'). Arrow indicates the position of ATP.

Polyacrylamide gel electrophoresis. Discontinuous polyacrylamide gel electrophoresis was conducted according to Ornstein [8] and Davis [9] in 5% gels. Approximately 0.06 units of enzyme were loaded and run at 5 mA per gel. After electrophoresis the gels were either stained with Coomassie Brilliant Blue R-250 or Bio Rad silver nitrate staining kit.

Protein determination. Protein content was measured as described by Bradford [10] using bovine serum albumin as the standard.

Results and discussion

Dystrophic male mice and controls (both 6–7 weeks old) were used to prepare the crude extract, as described under 'Materials and methods'. 5 g of tissue were homogenized in 50 ml final volume of three different buffers: 1) Tris-HCl 50 mM pH 7.6. 2) Tris-HCl 50 mM, pH 7.6 containing 1 mM PMSF. 3) Tris-HCl 50 mM, pH 7.6, containing 3 M KCl. The polyacrylamide gel electrophoretic pattern of the crude extracts from dystrophic muscle did not evidence any significant difference with respect to the controls, whatever the extraction buffer, even though such evidence cannot be conclusive (not shown). The highest content of total units of ATP:NMN adenylyltransferase activity was found when the extraction was conducted in the presence of 3 M KCl (table). NAD is the substrate of specific enzymes. One of these, the ATP:NMN adenylyltransferase, is present in the crude extract of skeletal muscle of dystrophic mice and its activity is about one half of that present in the muscles of normal mice (table).

Extracts prepared in Tris buffer without additions, or in the presence of 3 M KCl, or 1 mM PMSF, show that in the muscles of normal mice an appreciable quantity of ATP is present, in agreement with the results reported by Kushmerick [11], who found 6 and 5 μmoles/g w.wt of mouse extensor digitorum longus and soleus muscles respectively. In contrast, the same nucleotide is almost undetectable in the samples extracted from dystrophic skeletal muscle (figure). This evidence is very important because the NAD level is, at least in part, the result of the reaction catalyzed by ATP:NMN adenylyltransferase, where ATP is one of the substrates. Studies on NAD metabolism and the levels of the enzymes involved in the muscles of dystrophic mice and especially in cell cultures are in progress.

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Disruption of mitochondrial function as the basis of the trypanocidal effect of trifluoperazine on *Trypanosoma cruzi*

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Summary. The tricyclic anti-calmodulin drug trifluoperazine (TFP) inhibited growth and motility of epimastigotes of *Trypanosoma cruzi*, at concentrations lower than 100 μM, and motility and infectivity of the bloodstream trypomastigote form at 200 μM. Electron microscopy of TFP-treated epimastigotes showed that the major effect was at the mitochondrial level, with gross swelling and disorganization. The oligomycin-sensitive, mitochondrial ATPase was completely inhibited by 20 μM TFP, and the same drug concentration caused a 60% decrease in intracellular ATP content. The results suggest that the trypanocidal effect of TFP may be related more to mitochondrial damage than to the well-known anticalmodulin effect of the drug.

Key words. Trypanosoma cruzi; trifluoperazine; mitochondrial ATPase; trypanocidal drugs.