Identification of creatine as a cofactor of thiamin-diphosphate kinase

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Thiamin-diphosphate (TDP) kinase which catalyzes thiamin triphosphate formation from TDP requires a low-molecular-mass cofactor in addition to ATP and Mg^{2+}. The cofactor was isolated in a crystalline form from pig skeletal muscle and identified as creatine by proton NMR, mass spectrometry, infrared spectrometry and elemental analysis. The isolated cofactor and authentic creatine supported the same activity of partially purified TDP kinase at identical molar concentrations. Neither creatine phosphate nor creatinine showed activity as a cofactor. This is the first report showing evidence of the existence of a creatine-dependent enzyme.

Creatine *Cofactor* *Thiamin-diphosphate kinase* *Thiamin triphosphate*

1. INTRODUCTION

We have previously reported some properties of cytosolic thiamin-diphosphate (TDP) kinase which catalyzes the formation of thiamin triphosphate (TTP) from TDP in the presence of ATP, Mg^{2+}, and a cofactor of low-molecular mass [1]. Nishino et al. [2] reported another type of TDP kinase which catalyzes the formation of TTP from protein-bound TDP in the mitochondria of bovine brain. The activity of this mitochondrial enzyme depends also on a cofactor which was reported to be glucose [2]. Glucose at 5 mM did not, however, support the activity of our cytosolic TDP kinase [1]. The cofactor preparation used in [1] was trichloroacetic acid extract from guinea pig brain, which contained adenine nucleotides and other metabolites. This contamination prevented an exact analysis of the experimental results obtained, especially in the kinetic studies. This paper describes the purification and identification of the cofactor for cytosolic TDP kinase.

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2. MATERIALS AND METHODS

2.1. *Isolation of the cofactor*

Fresh pig skeletal muscle was obtained at a slaughterhouse and frozen at −70°C until use. One batch of skeletal muscle (50 g) was homogenized in 200 ml of distilled water in a Waring blender. The homogenate was centrifuged for 20 min at 10000 × *g*, then the supernatant was heated for 20 min in a boiling water bath and centrifuged again for 20 min at 10000 × *g*. The supernatant was concentrated in vacuo to 8.5 ml at 60°C. 1 vol. of chloroform/methanol (2:1) was then added, shaken and the chloroform layer discarded. The procedure was then repeated twice more. The water layer was then passed through Sep-Pak C_{18} (Waters) to remove yellowish pigments and the effluent concentrated in vacuo to 5 ml at 60°C. To 30 ml of water layer obtained from 6 batches of skeletal muscle, 6.0 g of Norit A were added to remove the thiamin phosphates and then centrifuged for 10 min at 10000 × *g*. The supernatant was concentrated in vacuo to 1 ml at 60°C and gradually chilled to 4°C to precipitate colorless crystals. The crystals were collected by

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filtrating through a glass filter, washed with ethanol and then ether. A crystalline cofactor of 145 mg, which was recrystallized 5 times from water, was obtained from 300 g of pig skeletal muscle.

2.2. *Analysis*

The proton NMR spectrum was recorded on a JEOL JNMGX-270 spectrometer in D_2O. Mass spectrometry was taken with a Shimadzu GLMS 7000S spectrometer. The infrared spectrum was measured with a Shimadzu IR 408 spectrometer. Elemental analysis was carried out with a Yanagimoto CHN recorder.

2.3. *Assay of TDP kinase activity*

TDP kinase activity was assayed as described in [1]. Partially purified (approx. 75-fold) TDP kinase was obtained from cytosolic fraction of pig skeletal muscle by ammonium sulfate precipitation (60–90% saturation), Sephadex G-75 gel filtration and affinity chromatography using Affi-Gel Blue (Bio-Rad).

3. RESULTS AND DISCUSSION

3.1. *Proton NMR spectrometry of the cofactor*

The proton NMR spectrum at 270 MHz of the cofactor preparation dissolved in D_2O is shown in fig.1. Two intense lines at 3.9 and 3.0 ppm were due to a methylene group and a methyl group, respectively. This spectrum was found to be nearly identical to the reference spectrum of creatinine in D_2O [3], suggesting that the cofactor is either creatinine or creatine.

3.2. *Mass spectrometry of the cofactor*

The EI mass spectrum of the cofactor shown in fig.2A reveals a prominent molecular ion at m/z 113, suggesting either creatinine or creatine hydrate. This was confirmed by the fact that authentic creatine and creatinine are identical in mass spectrum (fig.2B) and also identical to that of the cofactor (fig.2A).

3.3. *Infrared spectrometry of the cofactor*

The infrared spectrum of the cofactor was found to be identical to that of creatine (not shown).

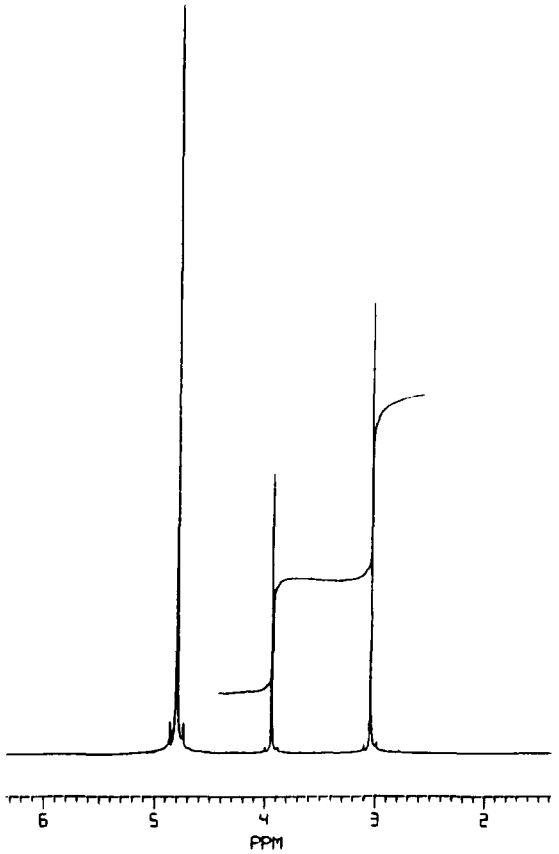

Fig.1. Proton NMR spectrum at 270 MHz of the cofactor dissolved in D_2O.

3.4. *Elemental analysis of the cofactor*

Elemental analysis revealed the percent of the components for the cofactor to be: C, 33.00; H, 6.20; N, 27.37. This result was compared with the component percentages calculated for creatine hydrate ($C_4H_{11}N_3O_3$): C, 32.21; H, 7.43; N, 28.18 and for creatinine ($C_4H_7N_3O$): C, 42.47; H, 6.24; N, 37.15. The values for creatine hydrate are very close to those found for the cofactor.

These results indicate that the cofactor required for the activity of cytosolic TDP kinase is very likely creatine hydrate.

3.5. *Biological activity of the cofactor isolated and of creatine*

The activity of the cofactor isolated was determined by assaying the activity of partially purified TDP kinase (pig skeletal muscle) which was dependent on the cofactor, and compared with that of

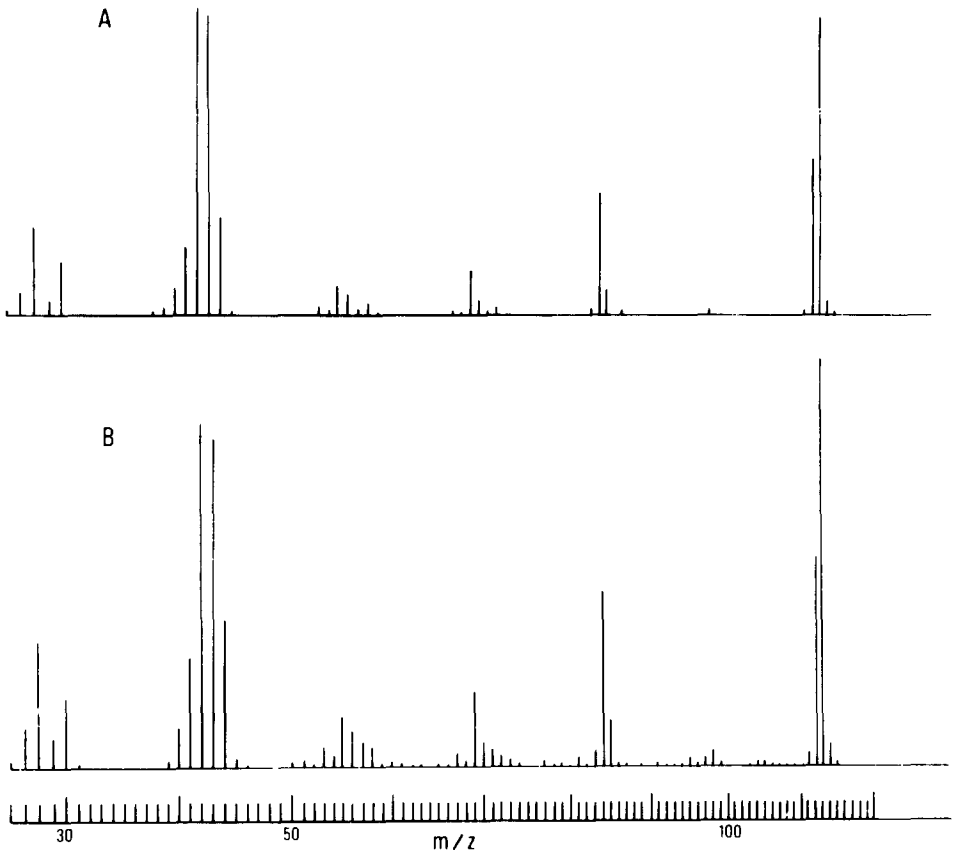

Fig.2. Mass spectra of the cofactor (A) and authentic creatine (B).

authentic creatine (fig.3). The cofactor and creatine supported the activity of TDP kinase in an identical dose-dependent manner. Creatine also supported the activity of guinea pig brain TDP kinase exactly as has been reported [1] (not shown). Saturation kinetics are shown with creatine (fig.3), and the apparent K_m value for creatine is calculated to be 0.2 mM, which is in physiological concentrations in various tissues [4,5]. Creatine phosphate was found to be completely inactive as a cofactor and as a phosphate donor to TTP synthesis by TDP kinase, and creatinine was inert as a cofactor (table 1). Creatine kinase (Boehringer Mannheim) showed no TDP kinase activity (not shown).

These results indicate that creatine is an essential cofactor of cytosolic TDP kinase. The cofactor required for TDP kinase activity in animal tissues [1] was isolated from pig skeletal muscle and identified as creatine (figs 1,2). Authentic creatine, but neither creatine phosphate nor creatinine, was found to give full activity of TDP kinase (fig.3, table 1). Other guanidino compounds (arginine,

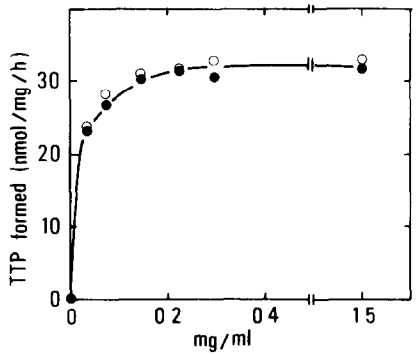

Fig.3. Titration of the cofactor and authentic creatine. TDP kinase activity was assayed in the presence of indicated concentrations of the cofactor (●) or authentic creatine (○) under the conditions described in the legend of table 1.

Table 1

Requirement of creatine for TDP kinase activity

Reaction mixture	% TDP kinase activity
Complete	100
− cofactor	0
− cofactor, + creatine	104
− cofactor, + Cr-P	0
− cofactor, + creatinine	0
+ Cr-P	109
− ATP	0
− ATP, + Cr-P	0

The complete reaction mixture contained in a final volume of 250 μl: 50 mM glycine buffer (pH 10.5), 0.1 mM TDP, 0.5 mM ATP, 0.5 mM $MgCl_2$, cofactor equivalent to 5 mM creatine, 0.1 mM dithiothreitol and 5 μg of enzyme preparation. Creatine, creatine phosphate (Cr-P) or creatinine was present at 5 mM when added. After incubation for 30 min at 25°C, TTP formed was determined as in [1]. The specific activity of TDP kinase was 59.9 nmol/mg protein per h

guanidinoacetic acid and guanidinopropionic acid) failed to show any cofactor activity (not shown). This is the first evidence which shows that cytosolic TDP kinase is a creatine-dependent enzyme. The mechanism of creatine involvement in TDP kinase reaction should be elucidated after the enzyme has been purified to a single protein. The fact that creatine concentration is high (7–15 mM) in the brain and skeletal muscle [4,5] and both tissues contain a high activity of TDP kinase [1], may suggest a role of TTP in neuro-muscular functions.

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