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ORIGINAL PAPER

Stabilization of NADH-dehydrogenase in Mitochondria by Guanosine Phosphates and Adenosine Phosphates

M. S. Frolova · N. L. Vekshin

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Abstract It is known that one of the reasons, leading to the development of neuromuscular diseases, including Parkinson's disease, is damage of the mitochondrial NADH-dehydrogenase. Perhaps, it happens when NADH-dehydrogenase loses connection with its coenzyme – flavine mononucleotide (FMN) that occurs at various influences on the enzyme. Previously, we have developed a method, based on fluorescence spectroscopy, to monitor the rate of exit of FMN from isolated mitochondria to solution. Also, we obtained the data that this process is blocked by the enzyme substrate – NADH or by the product – NAD. Recently, we found that this process is strongly blocked by adenine analogs of NAD, contained phosphates: ATP, ADP, and AMP. Adenosine phosphates are able to stabilize the FMN molecule in NADH-dehydrogenase. Using fluorescence spectroscopy and photocolorimetry, we have tested also other natural purine compounds - cAMP, cGMP, GMP, GDP, GTP, IMP, inosine, guanine, and caffeine. It is found that such derivatives of guanine as GMP, GDP, and GTP can prevent the release of FMN into solution. Guanine, cGMP, cAMP and caffeine did not prevent this process. The obtained data allow understand the mechanism of mitochondrial diseases, involving damage of mitochondrial NADH-dehydrogenase, and may help in development of medicines for treatment of these diseases.

Keywords NADH-dehydrogenase · Mitochondria · Neuromuscular diseases · Parkinson's disease · Mitochondrial diseases · Guanosine phosphates · FMN · Fluorescence spectroscopy · Flavine fluorescence

M. S. Frolova · N. L. Vekshin (✉)
Institute of Cell Biophysics, Institutskaja st., 3, 142290 Pushchino, Moscow region, Russia
e-mail: beornot@rambler.ru

Abbreviations

NADH	Nicotinamide dinucleotide
ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
FMN	Flavin mononucleotide
cAMP	Cyclic adenosine monophosphate
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GMP	Guanosine monophosphate
p-NTV	Para-nitro-tetrazolium-violet
UDP	Uridine diphosphate; CDP, cytosine diphosphate
CDP	Cytosine diphosphate

In recent years, a number of cases of age-related neuromuscular diseases are steadily grown in the world. One of the main reasons, leading to these diseases, is the disruption of the mitochondrial NADH-dehydrogenase – the most huge and important enzyme in respiratory chain [1, 2]. Perhaps, the disruption can be due that the NADH-dehydrogenase losses its coenzyme - FMN - a key link in the transfer of electrons from NADH to respiratory chain. As a result, a cell undergoes by oxidative stress and cannot work well.

The FMN molecule is located in a 50-kDa subunit of NADH-dehydrogenase (this subunit contains a pocket for binding of NADH). FMN is connected to the subunit by non-covalent bond and, therefore, the binding is unstable, especially under different treatments: temperature, detergents (0.001 %) and so on [3, 4]. FMN can be released even spontaneously, over time. As a result, a transfer of electrons from NADH to the electron transport chain will be blocked. In this case, electrons will be transmitted directly on molecular oxygen and transform it to superoxide anion. Overabundance of superoxide in cells leads to developing of oxidative stress, which may be a cause of many serious molecular diseases,

including neurodegenerative ills, such as Parkinson's disease and so on.

FMN is natural fluorophore and, therefore, it can be detected by own fluorescence, without using of special fluorescent probes. Under incubation of isolated mitochondria at 37 °C, the FMN molecule over a time (2 h) spontaneously releases from mitochondria and goes out into a free solution. This process is accompanied by increasing in the intensity of fluorescence and decreasing in the fluorescence polarization degree of FMN. Using this fluorescence spectroscopy approach, we can monitor the kinetics of releasing of FMN from mitochondrial NADH-dehydrogenase.

Previously, we have found that the process of releasing of FMN from NADH- dehydrogenase of mitochondria is prevented by NADH or NAD. Thus, NADH and NAD stabilize the enzyme due to their binding in the pocket of active center. When NADH or NAD penetrates into the binding site, the FMN molecule has not a way to pass through the pocket to aqueous phase. Unfortunately, NADH and NAD are hardly can be used as medicine preparations, since they are difficultly penetrate into cells and also they can hamper in a cell the functioning of others NAD- dependent enzymes.

In the presented study, we tested the ability of adenine derivatives (analogues of adenine part of molecule of NAD) and guanine derivatives (analogues of adenine derivatives) to stabilize FMN in NADH-dehydrogenase in isolated rat liver mitochondria, as a simple comfortable model object. In order to amplify the penetration of the tested substances into mitochondria, we used a moderate-hypotonic buffer, containing only 100 mM sucrose and 10 mM Tris-phosphate (pH 7.0). In this buffer, the mitochondrial membranes become more permeable. Also, we used the mitochondria which were defrosted once. One frosting-defrosting does not destroy organelles, but improves the permeability of their membranes and promotes better penetration for substances.

Enzymatic activity of NADH-dehydrogenase was determined by the colorimetric method of Belyakovich [5], using para-nitro-tetrazolium-violet (p-NTV), which is reduced to a red-colored formazane. This method make possible to measure the activity of NADH-dehydrogenase without respiratory chain blockers, because the speed of recovery of p-NTV to formazane by the enzyme is ten times higher than the rate of electron transfer in the mitochondrial electron transport chain.

Materials and Methods

Mitochondria from rat liver were isolated in medium, containing 20 mM Tris–HCl and 250 mM sucrose (pH 7.7) at 2 °C by standard method with some modifications [6]. First, the initial total suspension was centrifuged at BECKMAN J2- 21 at 1,000 g during 15 min. In result, cells and other large particles were sedimented and discarded. The supernatant was centrifuged during 15 min at 5,000 g, whereby are precipitated mature heavy mitochondria, which then were suspended in the same medium (9 ml per 1.5 ml of pellet) and was dosed into Eppendorf tubes by 1 ml, which were frozen and then were used for experiments (after defrosting).

In some cases, isolation of mitochondria was done in the medium, containing 200 mM sucrose, 100 mM mannitol, and 10 mM HEPES (pH 7.5). In this case, a second washing was performed with double centrifugation at 5,000 g.

The enzymatic reaction of NADH oxidation by p-NTV was detected by the appearance of colored formazan inside of mitochondria. Molecule of p-NTV is reduced to formazan during enzymatic oxidation of NADH, and therefore, the suspension of mitochondria becomes pink.

We added 100 mM NADH, 100 mM p-NTV and tested substances (GDP, guanosine etc.) in excess quantity (up to 300 μM) to suspension of mitochondria. The kinetics of increase in optical density due to formation of formazan was measured during 15 min with spectrophotometer PromEkoLab-5400UF (St. Petersburg) at 540 nm.

Concentration of mitochondrial protein was determined by UV-express method [7]. All mitochondrial fractions were aligned for protein content by buffer (100 mM sucrose and 10 mM Tris-phosphate, pH 7.0) to the final concentration of 0.3 mg/ml.

Spectra of flavine fluorescence were recorded with spectrofluorometer SLM-4800 (SLM, Inc., USA) in 1-cm or 0.4-cm cuvettes at 20 °C. Flavine fluorescence was excited at maximum of the absorption band at 450 nm and detection was done at maximum of the emission band at 525 nm. In some experiments, where fluorescence intensity was too low, in particular, in the case of measuring of polarization degree, the flavine fluorescence was registered in a special mirror cuvette, allowing amplify the signal at several times [4].

To detect the localization of FMN after 2 h of incubation (at 37 ° C), the suspension of mitochondria was passed through 0.2 μm Millipore filter, and then the flavine fluorescence was measured in the obtained filtrate (mitochondria are rest adsorbed on the filter). In addition to flavines and flavoproteins, also protomitochondria (small immature mitochondrial particles) could penetrate through 0.2 μm pores, but they have almost not flavines [8, 9] and therefore do not affect on measurements. In most experiments, mitochondria itself did not hinder on measurement of fluorescence of free FMN, and that is why they were not removed. All measurements were performed 5–10 times on the mitochondrial fractions, obtained from different animals, and then an average value was calculated.

Results and Discussion

Previously, we have shown that the process of output of FMN from the active center of mitochondrial NADH-

dehydrogenase can be accelerated by certain factors, in particular by a little quantity of detergents (about 0,001 %), as well as prolonged (1–2 h) keeping at moderate temperature (37 °C). This observation is in consistent with well-known fact about complete or partial loss of FMN during isolation and purification of the enzyme [10–14]. The loss of FMN is accompanied by a fall of NADH: ubiquinone reductase activity, but not NADH: ferricyanide reductase activity. This is related to the fact that the FMN molecule is not involved in the second reaction [4, 6]. Also, FMN is not involved in NADH: tetrazolium reductase reaction [4].

In the present study, we observed a spontaneous release of FMN from mitochondria after their incubation during 2 h at 37 °C. This process was accompanied by sufficient increasing in the intensity of flavine fluorescence and decreasing in polarization degree. The both parameters were measured at λex=450 nm and λem=525 nm at 20 °C. In addition, we measured a tryptophan fluorescence (λex=286 nm, λem=340 nm) to determine the output of proteins from mitochondria (most mitochondrial proteins contain tryptophan residues), and fluorescence of 7-aminoactinomycin (λex=530 nm, λem=620 nm) to determine the release of mitochondrial DNA (7-aminoactinomycin is well specifically integrated into the unwound DNA regions [4]). However, release of proteins and DNA from mitochondria into free solution was not observed: the intensity of tryptophan fluorescence and 7-aminoactinomycin fluorescence in solution after the 2 h incubation of mitochondria at 37 ° C and subsequent removal of mitochondria by 0,2-μm filters was negligible (data are not shown). This means that mitochondrial membranes remained generally intact.

Intensity of flavine fluorescence in control samples was enhanced after 2-h incubation by 2–3 times (it varies in different experiments, depending on the characteristics and degree of damage of mitochondria). There are two reasons for it, namely: 1) increasing in the absorption of the exciting light by FMN due to releasing from light-scattered particles, 2) increasing in fluorescence quantum yield of FMN due to change in the environment.

It should be noted that some contribution to the total flavine fluorescence of mitochondrial suspension gives flavine adenine nucleotide (FAD), which are tightly covalently associated with several mitochondrial dehydrogenases. But the *change* in the flavine fluorescence intensity belongs mainly due to release of FMN from NADH-dehydrogenase into solution. FAD molecules of mitochondrial flavoproteins do not give any significant contribution to the change of fluorescence, because FAD molecules remain covalently bound with their enzymes. Fluorescence quantum yield of FAD is 2 times low then FMN [4]. Besides, flavine fluorescence of mitochondria is low due to scattering of excitation light on each single mitochondrial particle, i.e. a number of photons cannot be absorbed by FAD. Also, the FAD fluorescence in mitochondria may be particularly quenched by aromatic amino acids and iron-sulfur clusters.

In addition to recording of flavine fluorescence intensity, we measured the flavine fluorescence polarization degree. This parameter gives us knowledge about rotation of FMN, i.e. a free flavine can be easy differently detected, separately of a bound flavine [4]. Polarization degree of the bound flavine is high due to a short lifetime of the excited state and low rotational mobility of the flavine within the protein. When FMN releases into aqueous solution, the polarization degree is decreased, because the velocity of rotation is greatly increased in aqueous phase (also, the lifetime is increased - due to disappearing of scattering of excitation light on the particles and due to lack of quenching by iron-sulfur clusters). In our experiments, the polarization degree of flavine fluorescence was decreased after incubation at 37 °C over 2 h to 1.5–2 times.

For checking the place, from where FMN emits - from mitochondrial matrix or from external solution - we measured the intensity and polarization degree of filtrates, which were obtained by filtration of mitochondrial suspensions (before and after 2-h incubation at 37 °C) through 0.2 μm Millipore filters. The intensity of fluorescence of the filtrate was strongly increased (after 2-h incubation of mitochondria) up to 4.5 times. The fluorescence polarization degree of the filtrate was reduced greatly - to 0.06, which corresponds to free rotation of FMN in water [4]. The obtained data clearly indicate that almost all FMN molecules are released (as a free form) from mitochondria to the external solution.

The FMN molecules, departed from iron-sulfur clusters (which can quench fluorescence of FMN in the NADH-dehydrogenase), in principle, may be also released from the enzyme to the mitochondrial matrix (if FMN has no time to get out into the external solution), but they give only a small contribution to changes of the intensity and polarization.

It is necessary to emphasize that flavine fluorescence *changes* in our experiments are not associated with a processes of oxidation/reduction of flavines, because incubation of mitochondria during 2 h in most of our experiments was performed in the absence of NADH, succinate and other reducing or oxidizing agents. Only in one case - the using of NADH - two phenomena during incubation took place at the same time: the reduction of fluorescent FMN to non-fluorescent FMN-H_2 and stabilization of FMN in the enzyme. Since the reduced form of flavine FMN-H_2 has disintegrating electron clouds and is unstable (electrons are rapidly transferred into respiratory chain), the FMN-H_2 quickly returns to FMN – the oxidized fluorescent form. Thus, it is unlikely that any red-ox transitions FMN/FMN-H_2 make a significant contribution to the observed *changing* of flavine fluorescence.

Previously [15], we tested as stabilizers of FMN in NADH-dehydrogenase of mitochondria the following group of substances: adenine, adenosine, ATP, ADP and AMP (they are

analogs of adenine part of NAD) and also nicotinic acid and nicotine amide (they are analogs of the nicotine part of NAD). The final data are shown in Table 1. The best stabilizers are AMP, ADP and ATP.

In the present study, we tested the more distant analogs of NAD: cAMP, caffeine, GMP, GDP, GTP, cGMP, UDP, CDP, inosine and IMP.

When adding of GTP, GDP and GMP at a concentration of 300 μM to the suspension of liver mitochondria was done with subsequent cultivation during 2 h at 37°C, the intensity of flavine fluorescence was increased in 1,6, 1,8 and 1,8 times, respectively (compared to the initial level, Fig. 1, Table 2). It is less in 2.4, 2.1 and 2 times comparing to the control. For comparison, the intensity of flavine fluorescence under the same conditions in the presence of ATP, ADP and AMP was changed lesser than in the control experiments, in 1.8, 2 and 1.7 times.

The btained data clearly shows that the used purine phosphates inhibit the release of FMN from NADH dehydrogenase of mitochondria and, therefore, stabilize the enzyme.

Flavine fluorescence polarization degree in the presence of GTP, GDP and GMP in comparison with the control sample (without adding substances) was greater in 1,7; 1,5 and 2 times, respectively (Fig. 2, Table. 2). This indicates that presence of these substances allow FMN to be remained in association with the enzyme.

We also tested how these substances affect on enzymatic oxidation of NADH. At Fig. 3, the kinetics of NADH:tetrazolium reaction for 15 min is shown. It is seen that in presence of guanosine phosphates the kinetics has some small decrease in the velocity at the end of the reaction (comparing to the control sample). It can be concluded that GTP, GDP and GMP (unlike of adenine nucleotides) depress at some small degree on the enzymatic activity of NADH-dehydrogenase (Fig. 3). In the used concentrations, they a bit compete with NADH for the active centre of the enzyme. The smallest influence on the reaction belongs to GMP. Therefore, GMP can be viewed as potential prototype for chemically synthetized analogs, which could be applied in medicine.

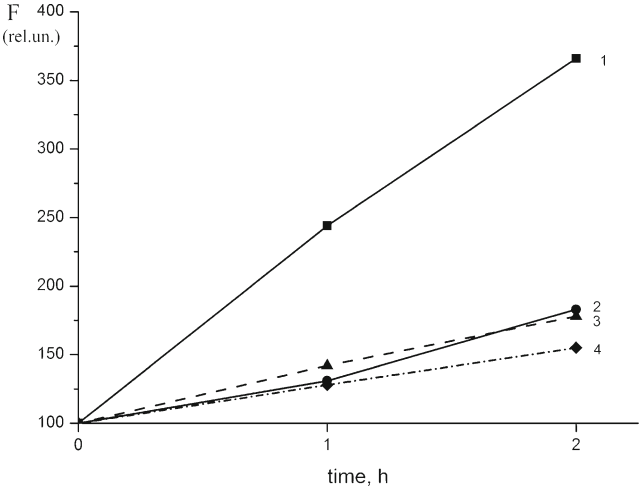

Fig. 1 Effect of guanosine phosphates on the intensity of flavine fluorescence during incubation of mitochondria at 37 °C after 2 h: 1 - control, 2 - GMP, 3 -GDP, 4 - GTP. The fluorescence intensity was measured at 20 °C

In order to clear the importance of phosphate groups in stabilization of the enzyme, we conducted the investigation with guanine and caffeine (these purines do not contain phosphate). Also, two pyrimidine di-phosphates - UDP and CDP – were tested.

When guanine or caffeine was added (in concentration of 300 μM) to suspension of mitochondria, the flavine

Table 1 Influence of NADH and its analogs (300 μM) on the intensity and polarization degree of flavine fluorescence of mitochondria during 2-h incubation at 37 °C. The initial intensity was taken as 100 %, the initial polarization degree was 0.32

The substance name	Intensity, %	Degree of polarization
Control	241	0,11
Adenine	307	0,18
Adenosine	256	0,2
AMP	142	0,2
ADP	122	0,21
ATP	133	0,22
NADH	199	0,21
NAD^2	185	0,25
Nicotine amid	336	0,15
Nicotinic acid	322	0,12

mitochondria were isolated by method 1 (see materials and methods)

Table 2 Influence of NADH and its analogs (300 μM) on the intensity and polarization degree of flavine fluorescence of mitochondria during 2 h incubation at 37 °C. The initial intensity was taken as 100 %, the degree polarization was 0.25

The substance name	Intensity, %	Degree of polarization
Control	366	0,11
Guanine	295	0,12
GMP	183	0,19
GDP	178	0,17
GTP	155	0,19
Caffeine	369	0,08
cGMP	348	0,15
cAMP	345	0,15
UDP	214	0,11
CDP	184	0,11
Inosine	366	0,09
Inosine phosphate	317	0,09

mitochondria were isolated with method 2 (see materials and methods)

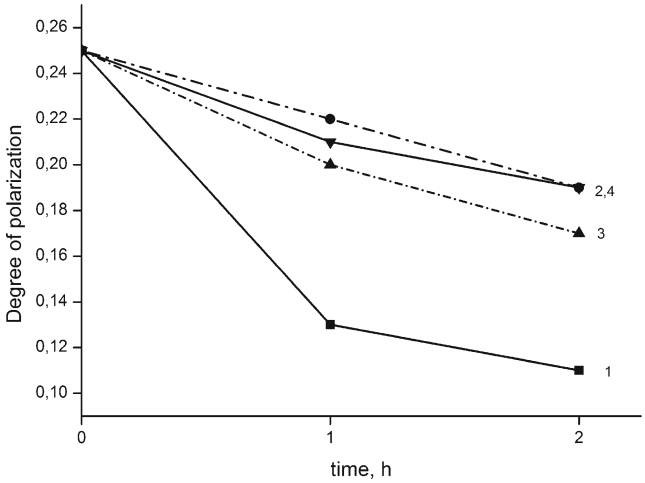

Fig. 2 Effect of guanosine phosphates on polarization degree of flavine fluorescence of mitochondria during incubation at 37 °C during 2 h: 1 - control, 2 - GMP, 3 - GDP, 4 - GTP. Polarization was measured at 20 °C

fluorescence intensity and polarization degree were changed like in the control sample (Table 2). Thus, it is no influence.

After addition of UDG or CDG to mitochondrial suspension under the same conditions, the intensity of flavine fluorescence was also changed like in the control (Table 2). Thus, it is no influence.

We have also tested cAMP and cGMP. In these cases also the intensity and polarization of flavine emission were changed as in the control sample.

The influence of inosine on the flavine emission of mitochondria was negligible. Action of inosine mono-phosphate (IMP) was not too expressed (Table 2).

Thus, these tested compounds are hardly could be used as potential stabilizers of NADH-dehydrogenase.

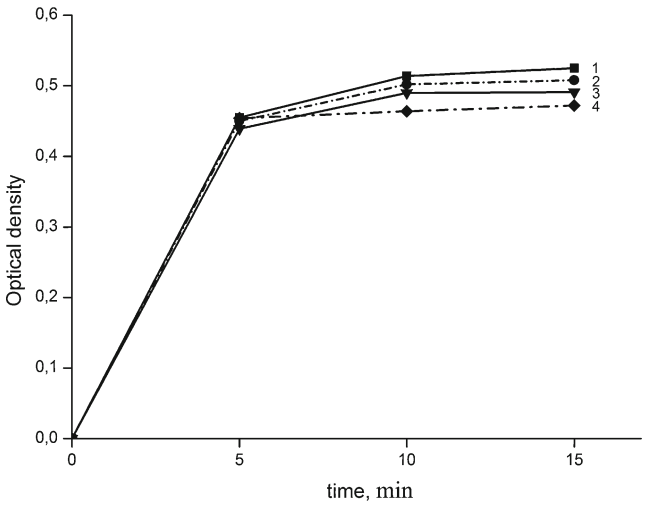

Fig. 3 Action of guanosine phosphates on the reaction of reduction of p-NTV to formazane in mitochondria (at 20 °C): 1 - control, 2 - GMP, 3 - GDP, 4 - GTP

A number of researchers [16–19] stated that the NADH-dehydrogenase has two binding site. It was assumed that NADH is oxidized to NAD in the first site, but NAD is reduced to NADH in the second one. If so, we can suggest a hypothesis that such analogues of NAD, which stabilize the molecule of FMN in the enzyme (GMP, GDP, GTP, AMP, ADP, and ATP), bind with the second center and stabilize it, mechanically preventing exit of FMN from the enzyme into solution (especially if the first center is occupied by molecule of NADH or NAD) (Fig. 4). Guanine phosphates and adenine phosphates did not compete with NADH for the first binding site, since binding constant for NADH, as well-known, is very high (~ 10^5 M^{-1}). That is why they do not inhibit the NADH:tetrazolium reaction even at 300 μM concentration. Obviously, guanine and adenosine phosphates interact with the second center or may be they have any own binding site in NADH-dehydrogenase.

In summary, we go to conclusion that guanosine phosphates (GTP, GDP, GMP) as well as adenosine phosphate (ATP, ADP, AMP) effectively prevent the release of FMN from NADH-dehydrogenase of mitochondria.

Because of adenosine phosphates and guanosine phosphates are involved in the energy metabolism, their real content in living cells depends on many biochemical processes. In particular, a balance between amount of phosphorylated and dephosphorylated derivatives may significantly vary, depending on the activity of ATPases and other enzymes. Introduction into cells an excess amount of adenosine phosphates or guanosine phosphates will not cause any disorder in

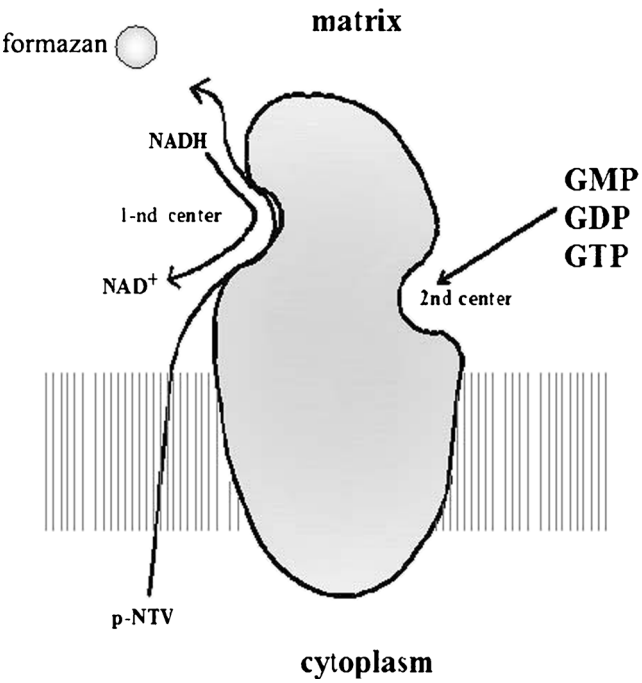

Fig. 4 Stabilization of FMN in mitochondrial NADH-dehydrogenase by guanosine phosphates

intracellular balance, but may temporarily stabilize the mitochondrial NADH-dehydrogenase.

It is no accident that AMP and ATP are currently used in sport medicine [20], as well as for the treatment of muscular dystrophy, chronic coronary insufficiency and myocardial dystrophy.

Guanosine phosphates are mainly involved in regulatory mechanisms. Therefore, they should be more carefully examined on their effects on the human body in possible treatment of neuromuscular diseases. Also, a number of chemical analogues of AMP and GMP could be synthetized in future and tested for prevention and treatment of mitochondrial aging and neurodegenerative diseases.

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