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Nucleotides of Beef Heart Mitochondria and Submitochondrial Particles

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Abstract

The composition and content of acid soluble nucleotides in intact beef heart mitochondria and different submitochondrial particles have been studied.

It has been established that phosphorylating $ETP_H(Mg^{++}, Mn^{++})$ and slightly phosphorylating ETP(KOH) fragments of inner mitochondrial membrane, which possess a similar morphological structure, have identical composition and almost the same content of acid soluble nucleotides. Both types of particles contain adenine nucleotides, nicotinamide and flavin coenzymes. The content of ATP in both types of particles amounts to 70-80! of that in intact mitochondria. Not less than three molecules of adenine nucleotides were found to be present in such particles per cytochrome *a* in a respiratory chain.

If mitochondria are sonicated in the presence of EDTA or $ETP_H(Mg^{++}, Mn^{++})$ is treated with urea, one can obtain non-phosphorylating particles of a quite different morphology and much lower content of adenine nucleotides. In the case of ETP_H (EDTA), only one molecule of adenine nucleotides remains bound with the respiratory chain. The conclusion may be drawn that, if the structure of submitochondrial fragments is damaged or changed, the content of nucleotides decreases sharply.

Introduction

It has been established that, besides oxidative phosphorylation, many other biochemical reactions run in mitochondria. Mitochondrial ATP synthesis is carried out by a chain of respiratory carriers, localized in the inner mitochondrial membrane and utilizing the products of the Krebs

cycle. The redox chain is characterized by a certain set of nucleotides and nucleotide coenzymes which are bound to it functionally. It was important to determine the content and composition of these compounds in the inner mitochondrial membrane, to ascertain the change in the nucleotide pool in the course of fragmentation of the organelles and also to find out whether the phosphorylating ability of the particles depends upon the content of the adenine nucleotides in them.

Nucleotides from beef heart mitochondria and four kinds of submitochondrial particles, differing morphologically and functionally, have been studied. This was an interesting question to study, for literature contains few data on the content of nucleotides in submitochondrial particles, and they are often only rough estimates mainly referring to the content of adenine nucleotides.

Methods

Isolation of Mitochondria and Submitochondrial Particles

Beef heart mitochondria were isolated as described by Crane *et al.* [1]. Separation of light and heavy mitochondria was performed in 0.25 M sucrose with 0.01 M Tris-HCl, pH 7.8. The heavy mitochondria were used for analysis or for preparation of submitochondrial particles differing in morphological structure or in the degree of coupling of oxidation and phosphorylation, i.e. phosphorylating submitochondrial particles—ETP_H (Mg^{++},Mn^{++}), ETP(KOH) and non-phosphorylating ones—ETP_H (EDTA) and ETP_H (urea).

Phosphorylating sonic submitochondrial particles ETP_H (Mg^{++},Mn^{++}) were isolated as described by Hansen and Smith [2] in the medium containing no ATP.

Morphologically similar, but slightly phosphorylating particles ETP were prepared by destruction of mitochondria swollen at pH 8.5 in a homogenizer with a Teflon pestle as described by Crane *et al.* [1].

ETP_H(EDTA) were isolated by the method of Beyer [3]. Mitochondria were sonicated in the presence of 2 mM EDTA at pH 7.8.

ETP_H (urea) were prepared from ETP_H (Mg^{++},Mn^{++}) by treating them with IM urea for one hour in the ATP-deprived medium [4].

All the preparations were suspended in 0.25 M sucrose; aliquots were taken to measure enzymic activities and to determine protein and cytochrome *a* concentrations. The resulting preparations were kept at $-20°$ during the night before extraction.

Characterization of the Preparations

ETP_H (Mg^{++}, Mn^{++}) were characterized by their phosphorylating ability. Alkaline and urea treated and EDTA particles were assayed as to their NADH oxidase, succinate oxidase and ATPase activities.

The phosphorylating ability of the ETP_H (Mg^{++}, Mn^{++}) was determined fluorometrically by recording the NADPH fluorescence at 460 nm in a sensitive spectrofluorimeter by means of the glucosehexokinase trap. The phosphorylating activity of the particles was 185 nmoles ATP/min/mg protein on NADH oxidation and 98 nmoles ATP/min/mg protein on succinate oxidation; the activity of reversed electron transfer was 216 nmoles NADH/min/mg protein.

The respiratory rate was measured spectrophotometrically in a Hitachi EPS-3 recording spectrophotometer. The succinate oxidase reaction was determined at 230 nm by the increase in fumarate concentration in the medium. All kinds of slightly or non-phosphorylating particles, ETP, ETP_H (EDTA) and ETP_H (urea), displayed similar succinate oxidase activities of about 2 μmoles/min/mg protein. The NADH oxidase activity determined as described in [4] was about 4 μmoles NADH/min/mg protein. The ATPase activity was determined by acidification of the incubation medium by means of a recording automatic pH-meter [5]. In the case of ETP_H (EDTA) and ETP_H (urea) it equalled 0.13 and 0.29 μg-H^+ ion/min/mg protein respectively.

Determination of cytochrome *a* concentration in preparations of mitochondria and submitochondrial particles was carried out by the method of Evtodienko and Mokhova [6] in a differential double-beam spectrophotometer.

Protein in the preparations of mitochondria and particles was determined by means of the method of Gornall *et al.* [7].

Analysis of Nucleotide Material

Acid soluble nucleotides were extracted from mitochondria and submitochondrial particles by cold 0.5 N perchloric acid. The extraction was repeated three times. Then the extract was neutralized by KOH and lyophilized as described earlier [8]. In the case of ETP_H (Mg^{++}, Mn^{++}) the nucleotides were sorbed on charcoal and eluted with 50% ethanol pH 8.5 to remove the ions of salts. Under such treatment, the loss of the nucleotide material amounted to 30%.

Analysis of nucleotides was made by ion exchange chromatography in a Hitachi 034 universal liquid chromatograph in system HCl-NaCl by the method described earlier [8].

Results and Discussion

Investigation of Nucleotides from Beef Heart Mitochondria

We have shown earlier that among the acid soluble nucleotides of beef heart mitochondria there are AMP, ADP, ATP, nicotinamide, flavin nucleotides and some small amounts of uridylic and guanylic acids

derivatives. Calculation of the nucleotide content per gramme of mitochondrial protein has shown that the average content of acid soluble nucleotides is 7.60 ± 0.36 nmoles/mg protein, the sum of adenine nucleotides is 4.76 ± 0.30 nmoles, NAD^+ + NADH is 1.47 ± 0.16 nmoles, $NADP^+$ + NADPH is 0.64 ± 0.09 nmoles, flavins—0.71 ± 0.03 nmoles [8].

A different system of calculation was required to establish what portion of the nucleotides of the whole mitochondrion is localized in its inner membrane. Cytochrome *a* is known to be localized in the inner membrane of mitochondria. Having calculated the amount of nucleotides in mitochondria and submitochondrial particles per cytochrome *a* unit, one may determine the share of any compounds of submitochrondrial fragments in relation to their content in intact mitochondria and besides, the number of nucleotide molecules associated with the respiratory chain.

The content of mitochondrial nucleotides per cytochrome *a* unit is given in Table I.

One may see that the average content of nucleotides in beef heart mitochondria is 21 nmoles per nmole cytochrome *a*, that of adenine nucleotides—about 13 nmoles (60% of the total), NAD^+ + NADH – 3.5, $NADP^+$ + NADPH – about 2.5 nmoles/nmole cytochrome *a*. The content of flavins approximately equals 1.5 nmoles/nmole cytochrome *a*. The summary content of uridylic, guanylic and inosinic acids does not exceed 1 nmole/nmole cytochrome *a* (about 3% of mitochondrial nucleotides):

TABLE I. Acid soluble nucleotides of mitochondria and submitochondrial particles* (nmoles/nmole of cytochrome *a*)

Compound	Mitochondria	ETP_H (Mg^{++}, Mn^{++})	ETP	ETP_H (EDTA)
AMP	7.43	0.48	1.26	0.24
ADP	3.76	1.35	1.06	0.51
ATP	1.54	1.02	1.26	trace amounts
AMP+ADP+ATP	12.73	2.85	3.58	0.75
NAD^++NADH	3.50	0.18	0.20	—†
$NADP^+$+NADPH	2.58	0.43	0.50	0.68
FMN+FAD	1.56	0.72	0.55	0.45
GMP+IMP	0.57	—	trace amounts	—
GTP+UTP	0.06	—	—	—
Sum of nucleotides	21.00	4.18	4.83	3.05
Nucleosides and bases	11.52	2.57	1.74	2.19
Total	32.52	6.75	6.57	5.24

* An average of 2-4 experiments.
† The NAD^+ + NADH was not determined, as the NADH peak contained much EDTA.

TABLE II. Acid soluble nucleotides in submitochondrial particles*
(nmoles/mg protein)

Compound	$ETP_H(Mg^{++}, Mn^{++})$	ETP	ETP_H(urea)	ETP_H(EDTA)
AMP	0.32	0.39	0.19	0.23
ADP	0.90	0.60	0.57	0.45
ATP	0.68	0.48	0.49	–
AMP+ADP+ATP	1.90	1.47	1.25	0.68
NAD^++NADH	0.12	0.20	0.10	– †
$NADP^+$+NADPH	0.28	0.15	0.23	0.37
Flavins	0.46	0.36	0.34	0.39
Sum of nucleotides	2.76	2.18	1.92	2.47
Nucleosides and bases	1.51	1.08	0.68	1.97
Total	4.27	3.26	2.60	4.44

* An average of 2-4 experiments.
† See note to Table I.

A Comparative Study of Nucleotides from Mitochondria and Submitochondrial Particles

The results of a comparative study of acid soluble nucleotides from mitochondria and inner membrane fragments are shown in Tables I and II. Table I shows the quantity of mitochondrial nucleotides and those from submitochondrial particles per cytochrome *a* unit. Table II sets forth the content of acid soluble nucleotides in the particles calculated per mg of mitochondrial protein.

One can see in Tables I and II that inner membrane fragments contain AMP, ADP and ATP, nicotinamide and flavin coenzymes. The content of guanine nucleotides is rather small and no uridine nucleotides were found. The content of nucleotides in the fragments amounts to approximately one fifth of those in mitochondria and ranges from 3 nmoles/nmole cytochrome *a* in ETP_H (EDTA) to 4-5 nmoles/nmole cytochrome *a* in ETP_H (Mg^{++}, Mn^{++}) and ETP.

The preparations under study were fragments of the inner membrane which differed in the degree to which the morphological structure has been preserved and to which the oxidation and phosphorylation is coupled. Hence, it was interesting to find out whether there was any relationship between the composition and content of acid soluble nucleotides and the phosphorylating ability of the particles.

For example, ETP_H (Mg^{++}, Mn^{++}) which possess all the components of the respiratory chain proved capable of coupling oxidation and phosphorylation, reversed electron transfer and transhydrogenase reaction. An electron microscopy study showed these particles to have characteristic repeating subunits on their outer surface. Apparently,

these inner membrane fragments maintain the structure necessary for normal energy transformation.

A study of the content of the acid soluble nucleotides in these particles proved them to contain about 40% of ADP and 70% of ATP of intact mitochondria. These results are yet another proof that the inner membrane of mitochondria has the leading role in the mitochondrial ATP synthesis. The total content of adenine nucleotides is about 3 nmoles/nmole cytochrome *a*, or three molecules per respiratory chain. This suggests that this portion of adenine nucleotides is more or less firmly bound with the oxidative phosphorylation system.

One can see in Table II that the content of adenine nucleotides in the particles is about 2 nmoles/mg protein. These data are similar to Klingenberg's results for sonic submitochondrial particles from beef heart and also rat liver and heart [9].

In ETP_H (Mg^{++}, Mn^{++}) there remains about 0.6 nmoles of nicotinamide nucleotides/nmole cytochrome *a*. The content of acid soluble flavins amounts to 0.7 nmoles/nmole cytochrome *a* which is more than half of acid soluble flavins of mitochondria.

The inner membrane fragments obtained by disruption of the mitochondria at pH 8.5 are morphologically similar to ETP_H (Mg^{++}, Mn^{++}). However, this treatment seems to cause greater degradation of the membrane structure, which is expressed in that the particles may carry out only electron transfer and the transhydrogenase reaction, the process of coupling of oxidation and phosphorylation being very weak.

A comparison of the content and composition of nucleotides in the morphologically similar ETP_H (Mg^{++}, Mn^{++}) and ETP shows these preparations to have an identical nucleotide pool. As is shown in Tables I and II the fragments studied contain almost the same quantities of these compounds. In ETP there are also localized about 30% of ADP and 80% of ATP of the intact mitochondria. The adenine nucleotides content in these fragments is also about 3 nmoles/nmole cytochrome *a*.

The content of $NAD^+ + NADH$ and $NADP^+ + NADPH$ in these preparations is not high and almost similar. It should be noted that ETP contains somewhat less flavins in comparison with ETP_H (Mg^{++}, Mn^{++}).

Thus, ETP_H (Mg^{++}, Mn^{++}) and ETP, possessing similar morphological structures, have nucleotides with almost identical content and composition.

Treatment of ETP_H (Mg^{++}, Mn^{++}) with urea does not damage their respiratory chain. The particles prepared in this way carry out electron transfer and the transhydrogenase reaction. As is seen in Table II, the total content of nucleotides in these particles is less than in ETP_H (Mg^{++}, Mn^{++}) and ETP. In the urea-treated particles there are less adenine nucleotides than in the phosphorylating sonic and slightly phosphorylating ETP, i.e. about 1.25 nmoles/mg protein. The content of nicotinamide and flavin coenzymes is practically the same.

Sonication of mitochondria in the presence of EDTA, which is apparently the most rigid treatment damaging the structures, results in formation of the particles devoid of knobs. These particles are capable of electron transfer only. This treatment of mitochondria entails a decrease in the adenine nucleotide content in the resulting preparations (see Tables I and II), which is four times less as compared to the phosphorylating submitochondrial particles. Only trace amounts of ATP were found in these particles. This is in good agreement with the results of Beyer [10]. Thus, in these particles there is only one molecule of adenine nucleotides—and not three as in the case with ETP_H (Mg^{++}, Mn^{++}) and ETP per respiratory chain. On the other hand, the content of $NADP^+$ + NADPH is close to that in ETP_H(Mg^{++}, Mn^{++}) and ETP. The content of NADH in these particles was not determined as its peak contained EDTA. It should be noted that these particles contain more nucleosides and bases than others.

Conclusion

We believe that the study of the composition and content of nucleotides in mitochondria as compared to submitochondrial particles allows the following conclusions to be made.

1. The inner membrane fragments with the best preserved morphological structure, ETP_H (Mg^{++}, Mn^{++}) and ETP, have similar content and identical composition of the related acid soluble nucleotides. These particles characteristically possess the nucleotides which participate in the processes of oxidative phosphorylation, i.e. adenine nucleotides, nicotinamide and flavin coenzymes. Some of them are localized almost completely in the inner membrane of the mitochondria. This is ADP, ATP, FMN, FAD. Nicotinamide coenzymes are bound to the membrane less firmly. No uridylic and cytidylic acid derivatives were found in the fragments. Only trace amounts of guanine nucleotides were detected. This gives grounds to believe that such a nucleotide pool is characteristic of the whole inner mitochondrial membrane, in which the respiratory chain is localized and the electron transfer is coupled with ATP formation.

2. The calculation of the quantity of adenine nucleotides/cytochrome *a* shows that the best preserved morphologically inner membrane fragments have at least three molecules of adenine nucleotides per respiratory chain (assuming that one molecule of cytochrome *a* is localized in the respiratory chain). The damage to the morphological structure of the fragment inflicted by sonic treatment in the presence of EDTA or urea treatment results in a decrease (a very sharp one in the case of EDTA) in the adenine nucleotide content. In this case only one molecule of adenine nucleotides remains associated with the respiratory chain.

3. The action of EDTA is apparently that of breaking the coordination bonds *via* metallic ions which bind, directly or indirectly, the nucleotides and the elements of the membrane. One may think that adenine nucleotides are released in the solution not in the free state, but as large complexes. This agrees with the hypotheses of Skulachev [11, 12], Cooper [13] and Chapell and Crofts [14] about the adenine nucleotides functioning in the bound state in the oxidative phosphorylation process. Racker [15] also put forward the idea of the possible formation of a complex between ADP or ATP and coupling factor F_1 in the process of oxidative phosphorylation. There is some experimental evidence substantiating the high affinity of mitochondrial adenosine triphosphatase for ADP [5], and also the presence of ADP firmly bound to ATPase in mitochondria [16]. It is not impossible that the linkage between adenine nucleotides and enzymes in such complexes could be covalent. This possibility is supported, for example, by AMP being covalently bound to the tyrosine residue in the molecule of glutamine synthetase [17], FAD being bound with the molecules of succinate dehydrogenase [18] and monoaminooxidase [19]. These facts may be interpreted to mean that in our experiments a part of the adenine nucleotides could be removed from the particles together with the knobs, i.e. with ATPase. It is also possible that, when the inner membrane is treated with EDTA or urea, the carriers of adenine nucleotides (translocase), with which these compounds could be bound, are removed [20].

4. The phosphorylating ability of submitochondrial particles seems to depend not so much on their nucleotide pool but rather on the degree to which the inner structure of the fragments has been preserved. The content of adenine nucleotides in phosphorylating ETP_H and slightly phosphorylating ETP is similar. And only great damage to the membrane structure in the case of ETP_H (EDTA) and ETP_H (urea) and the loss of the ability to phosphorylate is accompanied by a sharp decrease in the adenine nucleotide content.

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References

1. F. L. Crane, J. L. Glenn and D. E. Green, *Biochim. Biophys. Acta*, 22 (1966) 475.
2. M. Hansen and A. L. Smith, *Biochim. Biophys. Acta*, 81 (1964) 214.

3. R. E. Beyer, in: *Methods in Enzymology*, vol. X. R. W. Estabrook and M. E. Pulman (eds.), Acad. Press, New York, 1967, p. 186.
4. V. N. Lusikov, V. A. Saks and I. V. Beresin, *Biochim. Biophys. Acta*, 22 (1970) 16.
5. V. K. Akimenko, I. B. Minkov and A. D. Vinogradov, *Biokhimiya*, 36 (1971) 655.
6. Yu. Evtodienko and E. N. Mokhova, in: *Mitochondria, Structure and Function*, Moscow, "Nauka", 1966, p. 95.
7. A. D. Gornall, C. G. Bardawill and M. M. David, *J. Biol. Chem.* 177 (1949) 751.
8. V. I. Drobishev, S. E. Mansurova and I. S. Kulaev, *J. Chromatogr.* 69 (1972) 317.
9. M. Klingenberg, in: *Mitochondrial Structure and Compartmentation*, E. Quagliariello, S. Papa, E. C. Slater and J. M. Tager (eds.), Adriatica Editrice, Bari, 1967, p. 320.
10. R. Beyer, *Canad. J. Biochem.*, 46 (1968) 677.
11. V. P. Skulachev, *Uspekhi Biologicheskoy khimii*, 6 (1964) 180.
12. V. P. Skulachev, *Energy Accumulation in the Cell*, Moscow, "Nauka", 1969.
13. C. Cooper, *Biochemistry*, 4 (1965) 335.
14. J. B. Chappel and A. R. Crofts, *Biochem. J.*, 95 (1965) 393.
15. E. Racker, *Mechanisms in Bioenergetics*, Acad. Press, New York and London, 1965.
16. J. B. Warshaw, K. W. Lam, B. Nagy and D. R. Sanadi, *Arch. Biochem. Biophys.*, 123 (1968) 385.
17. H. Holzer and W. Duntze, *Ann. Rev. Biochem.*, 40 (1971) 345.
18. W. H. Walker and T. P. Singer, *J. Biol. Chem.*, 245 (1970) 4224.
19. E. B. Kerney, J. I. Salach, W. H. Walker, R. Seng and T. P. Singer, *Biochem. Biophys. Res. Commun.*, 42 (1971) 490.
20. M. Klingenberg, *FEBS Letters*, 6 (1970) 145.