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Minireview

Mitochondrial carrier proteins

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Abstract

Ten mitochondrial carriers have been purified from animal mitochondria. They are small proteins with a molecular mass ranging from 28 to 34 kDa on SDS-PAGE. So far, five of these proteins have been sequenced. Their polypeptide chain consists of three tandemly related sequences of about 100 amino acids. The repeats of the different proteins are related and probably fold into two transmembrane α-helices linked by an extra-membrane loop. The features of this family are also present in several proteins of unknown function characterized by DNA sequencing. Isoforms of some carriers have been found. All mitochondrial carriers investigated in proteoliposomes function according to a simultaneous (sequential) mechanism of transport. The only exception is the carnitine carrier that proceeds via a ping-pong mechanism. Three mitochondrial carriers have been expressed in yeast and two overexpressed in *E. coli* and refolded in active form.

Key words: Carrier protein; Transmembrane topology; Bacterial expression; Transport; Liposome; Mitochondrion

1. Introduction

The transport of metabolites across the inner mitochondrial membrane is catalyzed by specific carrier proteins that span the lipid bilayer. At present there are at least twelve metabolite carriers well characterized in intact mitochondria [1,2]. The majority of these carriers deal with anions (ADP/ATP, P_i, oxoglutarate, aspartate/glutamate, pyruvate, citrate, dicarboxylates, branched keto acids and ATP Mg/P_i), but some transport cations or zwitterions (acylcarnitines/carnitine, ornithine, spermine and glutamine). The uncoupling protein (UCP) from brown fat mitochondria, which is a H^+ carrier, must also be included among these proteins because of the sequence similarity. In addition to these 'major' carriers, many others must exist in the inner mitochondrial membrane for the import of various nucleotides, cofactors and compounds which are not synthesized in mitochondria.

Nearly all the mitochondrial carriers characterized so far catalyse the exchange of substrates across the membrane. Those mediating H^+-compensated unidirectional substrate flux (PiC, PYC, GC) may also fall into the above category because at least the PiC has been shown to function in a phosphate/OH^- antiport mode [3]. The CAC is the only mitochondrial transporter that catalyzes both acylcarnitine/carnitine antiport and substrate uniport, whereas for the UCP the uniport mode is the exclusive transport mode.

The carriers involved in oxidative phosphorylation (AAC and PiC) are present in all mitochondria. Also the main carriers for import of reducing equivalents or substrates for oxidative phosphorylation in the mitochondrial matrix (PYC, CAC, OGC and AGC) are widely distributed. Other carriers, on the other hand, are tissue specific and have a limited distribution reflecting their importance in special functions, e.g. gluconeogenesis (DIC), fatty acid and lipid synthesis (CIC), urea synthesis (ORC, GC and GNC) or thermogenesis (UCP). Although characteristic of the inner mitochondrial membrane, the metabolite carriers are generally present in the membrane in very minute amounts. Only the AAC, the PiC and the UCP of brown fat represent an important share of the mitochondrial protein. As reported below, significant advancement in the field of mitochondrial metabolite transport systems has been made by elucidating an increasing number of primary structures, investigating the transmembrane topology, purifying and reconstituting all the major carriers, elucidating their kinetic mechanism, and expressing some of them in yeast and *E. coli*.

2. Identification and purification

Purification and subsequent reconstitution of pure

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Abbreviations: AAC, adenine nucleotide carrier; AGC, aspartate/glutamate carrier; CAC, carnitine carrier; CIC, citrate (tricarboxylate) carrier; DIC, dicarboxylate carrier; OGC, oxoglutarate carrier; ORC, ornithine carrier; PiC, phosphate carrier; PYC, pyruvate (monocarboxylate) carrier; UCP, uncoupling protein, H^+ carrier; GC, glutamate carrier; GNC, glutamine carrier.

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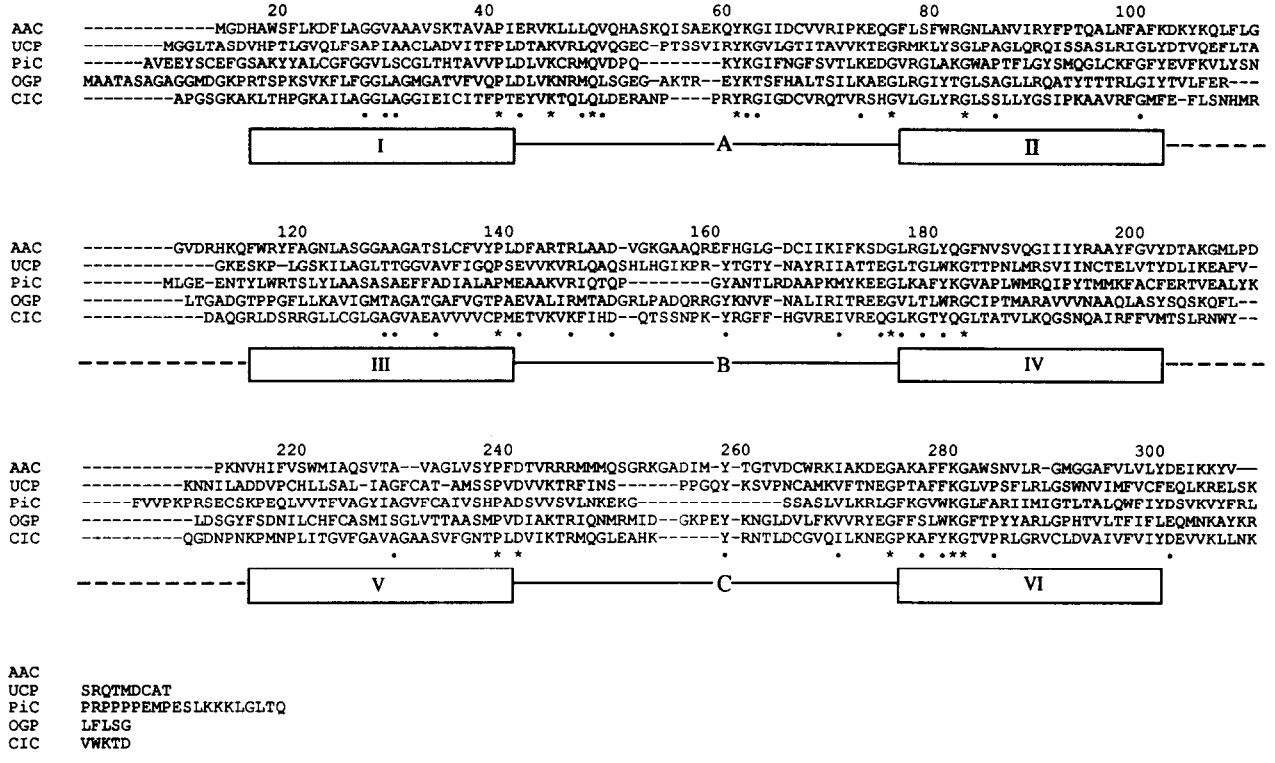

Fig. 1. Alignment of the repetitive elements in the primary structure of the biochemically known mitochondrial carriers sequenced so far. The asterisks indicate identities, while highly conservative substitutions are shown by the dots. The sequences shown are the human T1 isoform of AAC [23], the human UCP [24], the human PiC [25], the human OGC [26] and the rat CIC [19]. Segments I–VI represent hydrophobic regions that might be folded into transmembrane α-helices. The transmembrane segments are linked by extensive hydrophilic regions (A, B and C) and the three repeats by shorter stretches of hydrophilic amino acids (a′ and b′) indicated by the dotted lines.

protein in artificial membranes is essential for identification, detailed functional characterization and structural studies of a transport protein. So far, 10 carrier proteins have been purified to homogeneity from mammalian mitochondria [1,4]. Table 1 gives an overview of the purification of these carriers. The most striking feature is the particular usefulness of hydroxyapatite and celite in chromatographic procedures. With very few exceptions, all mitochondrial carrier proteins have been purified using variations of a general procedure based on hydroxyapatite and celite chromatography. All mitochondrial metabolite carriers isolated until now fall into a very

Table 1
Purification and reconstitution of mitochondrial carriers

Carrier	Source	Solubilization	Purified by	Mol. wt. (SPAGE)	Identification by	References
1. ADP/ATP	BHM	TX-100	HA-chrom.	30 kDa	Inhib. binding	[5]
2. Uncoupling protein	BFM	TX-100	HA-chrom./ultracentr.	32 kDa	GTP-binding	[6]
3. Phosphate	PHM	TX-100 + DPG	HA/affin. chrom.	33 kDa	Reconstitution	[7]
4. Oxoglutarate	PHM	TX-114	HA/celite chrom.	31.5 kDa	Reconstitution	[8]
5. Dicarboxylate	RLM	TX-114	HA/celite chrom.	28 kDa	Reconstitution	[9]
6. Citrate	RLM	TX-100 + DPG	HA/celite chrom.	30 kDa	Reconstitution	[10]
7. Pyruvate	BHM	TX-100	HA/affin. chrom.	34 kDa	Reconstitution	[11]
8. Carnitine	RLM	TX-100	HA/celite chrom.	32.5 kDa	Reconstitution	[12]
9. Aspartate/glutamate	BHM	$C_{12}E_8$	HA/celite chrom.	31.5 kDa	Reconstitution	[13]
10. Ornithine	RLM	TX-100	HA-chrom. and others	33.5 kDa	Reconstitution	[14]

Only the first publications reporting complete purification are mentioned. Abbreviations for different sources of mitochondria are: BHM, beef heart; BLM, beef liver; RLM rat liver; PHM, pig heart; BFM, brown fat. Other abbreviations: $C_{12}E_8$, octa-ethyleneglycolmono-n-dodecyl ether; DPG, cardiolipin; HA, hydroxyapatite; SPAGE, SDS-polyacrylamide gel electrophoresis.

narrow range of apparent molecular masses between 28 and 34 kDa (Table 1).

3. Primary structures and extension of the carrier family

The amino acid sequences of 5 mitochondrial carriers that have been biochemically characterized, namely AAC, UCP, PiC, OGC and CIC, have been determined by amino acid analysis or DNA sequencing [15–19]. The first primary structure of AAC from bovine heart was determined in 1982. During the last 10 years, AAC has been sequenced from 13 organisms (20 sequences including isoforms), UCP from 6, PiC from 5 (7 sequences including isoforms) and OGC from 4 species (see [20,21] for references, and [22]). Comparison of the amino acid sequences of the mitochondrial carriers with themselves has shown that they have a tripartite structure, made up of related sequences about 100 amino acids in length (Fig. 1). Each repetitive element contains 2 hydrophobic stretches (I–VI) separated by an extensive hydrophilic region (A–C), and the repeats of one carrier are related to those present in the others.

The alignment of 15 sequences of five functionally characterized carriers (Fig. 1) shows that 14 residues are totally conserved and 30 are substituted in a highly conservative manner. A distinct feature is the sequence motif P-h-D/E-h-h-K/R-h-R/K-(20–30 amino acids)-D/E-G-(4 amino acids)-a-K/R-G (h = hydrophobic, a = aromatic) which is present in many repeated domains. Although the number of conserved amino acids is relatively low, the tripartite structure, the presence of the two hydrophobic regions in each domain and the three-fold repetition of the sequence motif show that these five mitochondrial carriers belong to the same protein family. Their structure originates from a common ancestor by two-tandem gene duplication.

The characteristic sequence features of the mitochondrial carrier family have been used to identify other proteins of unknown function as members of the same family, such as MRS3 and MRS4 from *S. cerevisiae* (probably isoforms of the same protein), a protein associated with Grave's disease, a protein from the ciliated protozoa *Oxytricia fallax*, two proteins encoded by genes in chromosomes IX and XI from *S. cerevisiae*, and four proteins of the nematode *Caenorhabditis elegans* (see [20] for references). Recently, two other members of the mitochondrial metabolite carrier family, namely the maize brittle1 protein [27] and the PMP47 from *Candida boidinii* [28] have been identified in amyloplasts and peroxisomes, respectively, indicating that metabolite carrier proteins of this type are not restricted to mitochondria

Table 2
Kinetic data of purified and reconstituted mitochondrial carrier proteins*

Carrier	Substrate	K_m (mM) external	K_m (mM) internal	V_{max} (mmol/min × g)	Activation energy	Kinetic mechanism
AAC	ATP	0.01	0.025	up to 7.1	160/62	n.d.
	ADP	0.01				
PiC	P_i/P_i	1.8 ± 0.1	9.4 ± 0.1	92	64	Simultaneous
	P_i/OH^-		11.2 ± 0.9	53		Simultaneous
OGC	Oxoglutarate	0.31 ± 0.08	0.17 ± 0.06	9.5 ± 1.5	54	Simultaneous
	Malate	1.36 ± 0.14	0.71 ± 0.18	10.0 ± 1.1		Simultaneous
DIC	Malate	0.49 ± 0.05	0.92 ± 0.16	6.0 ± 1.6	96	Simultaneous
	Malonate	0.54 ± 0.10	n.d.	5.9 ± 0.9		n.d.
	Phosphate	1.41 ± 0.35	0.93 ± 0.18	6.0 ± 1.4		Simultaneous
CIC	Citrate	0.032 ± 0.004	0.027 ± 0.004	10.5 ± 1.9	70	Simultaneous
	Malate	0.25 ± 0.03	0.060 ± 0.006	11.5 ± 1.8		Simultaneous
AGC	Aspartate	0.12 ± 0.02	2.8 ± 0.6	n.d.**	77	Simultaneous
	Glutamate	0.25	3			Simultaneous
CAC	Carnitine (exchange)	1.1 ± 0.1	10 ± 0.8	1.7 ± 1.3	133	ping-pong
	Carnitine (uniport)	0.53 ± 0.12	2.2 ± 0.4	0.2 ± 0.09	115	
ORC	Ornithine	0.16 ± 0.02	4	3.2 ± 0.7	89	n.d.
PYC	Pyruvate	0.24 ± 0.02	n.d.	114 ± 21	n.d.	n.d.

* All data are from studies using hydrophobic chromatography for reconstitution except those for the AAC (see [4] and [43] for references and [3,44]).
** V_{max} value has not been determined with the purified protein (see [13]).

but represent a more extended family in eukaryotes. Including these new members, there are at present 16 different members of this protein family.

4. Secondary structure and transmembrane topology

The two hydrophobic stretches present in each of the three repeats are sufficiently long to span the membrane as α-helices. On the basis of the tripartite structure and the hydrophobic profile of AAC, Saraste and Walker [29] proposed that each repetitive domain consists of two transmembrane α-helices forming a structure with, in total, six α-helices (I–VI). The two helices of the individual elements are linked by an extensive, more polar segment (A, B and C), whereas the three repeats are connected by shorter stretches of hydrophilic amino acids (a′ and b′) (see Fig. 1). The essential predictions of this model are: the N- and C-termini of the polypeptide chain are exposed to the same side of the membrane, the three long hydrophilic segments connecting the two α-helices of each domain (A, B and C) are located on one side of the membrane (opposite to that of the N- and C-termini), and the two shorter hydrophilic segments a′ and b′ on the other side.

Many of these features have been demonstrated by investigating the accessibility of extra-membranous regions of various carriers to impermeable reagents such as peptide-specific antibodies and proteolytic enzymes. In the case of PiC, the N- and C- terminal regions as well as loops a′ and b′ are exposed to the cytoplasmic side of the inner mitochondrial membrane, whereas loop B is exposed to the matrix side [30]. Similarly, in the case of UCP, the N- and C-termini and loop a′ are oriented towards the cytosol, whereas loops A, B and C are oriented towards the matrix [31,32]. It was also shown that the N-terminal region of the AAC is exposed on the cytosolic side of the membrane [33], and when the carrier is conformationally stabilized by the inhibitor bongkrekic acid, the postulated loops A, B and C are accessible to proteases on the matrix side [34].

Recent topological data concerning OGC are also in agreement with the six α-helix model proposed by Saraste and Walker. Also in this case, the cytoplasmic exposure of both N- and C-terminal sequences was demonstrated, and furthermore loops A and B were shown to protrude into the matrix and loop a′ to be exposed towards the cytosol [30,35]. It is possible that during the conformational change occurring in the transport process one or more of the loops A, B and C might be inserted into the lipid bilayer as hairpin structures. It was shown that the segments of the polypeptide chain of AAC spanning residues F153–M200 and Y250–M281 (belonging to the matrix loops B and C in the model) are covalently labelled by two non-permeant specific ligands, azido atractyloside and azido [α-^{32}P]ADP, respectively, when these are added from the cytoplasmic side of the membrane [21]. Similarly, loop C of UCP was shown to be photolabelled by 2-azido ADP [36] and loop A of PiC to react, at the level of Cys-41 (rat liver PiC), with the impermeable eosin maleimide [37].

5. Genes and organ specificity

The sequences of the genes of AAC, UCP, OGC and PiC have been determined during the last years [22–24,26,38,39]. The structures of these genes are related, containing the introns in equivalent positions. For example, they all contain an intron after the first and before the sixth transmembrane segment. In this respect there is a tendency for the introns to interrupt the coding sequence in or near the extra-membrane loops.

Some mitochondrial carriers have isoforms encoded by different genes (see [20] for references, and [40,41]). The three human genes for AAC are expressed in heart, liver and fibroblasts, respectively. Only one gene, on the other hand, has been found for UCP and OGC. Although only one gene exists for PiC, two different transcripts have been characterized in man, cow [22] and rat (V. Iacobazzi and F. Palmieri, unpublished results). These arise from alternative splicing affecting amino acids 4–45 of the mature PiC protein [22]. One form is expressed most strongly in heart and liver, and the other in lung. The functional difference of the expressed isoforms in different mammalian tissues is not yet known. For the yeast AAC1 and AAC2 isoforms, it has been found that the transport activity of the former is 40% of that of the latter [42].

6. Functional studies

For functional characterization in terms of transport activity, the purified carrier proteins are reconstituted into liposomes. For eight purified and reconstituted carriers, a complete set of kinetic data, including substrate specificity, affinity, number of binding sites, interaction with inhibitors, activation energy, influence of pH and of phospholipids, has been obtained [4]. In particular, K_m values have been determined not only on the external side (as generally carried out in mitochondria) but also on the internal side of the proteoliposomes. Some of these data are summarized in Table 2. Importantly, for at least one substrate of each carrier, the apparent K_m on the inside is significantly different from that on the outside. Furthermore, except for AAC, no significant amount of the carrier population with 'internal K_m' was found on the outside and vice versa. Most carriers are, therefore, unidirectionally inserted into the liposomal membrane. By comparing the internal and the external transport affinities determined in proteoliposomes with

the values measured in intact mitochondria, it has been concluded that AGC, CIC, DIC, CAC and PiC are oriented right-side-out, as in intact mitochondria, whereas the OGC is inserted in the opposite direction (see [4] for references).

The asymmetric orientation of the membrane-embedded carrier proteins, and the independence of the exchange reactions from the type of counter-substrate have provided well-defined conditions for studying the transport mechanisms of the reconstituted mitochondrial carriers using a kinetic approach. Two-substrate initial velocity studies showed that the reconstituted AGC, OGC, CIC, DIC and PiC function according to a simultaneous (sequential) mechanism, implying that one internal and one external substrate molecule form a ternary complex with the carrier before transport occurs (see [4]). A sequential mechanism has also been shown for OGC and AGC by studies with intact mitochondria [45,46].

On this basis it has been proposed that the mitochondrial carriers not only form a structural family but also constitute a homogeneous functional family characterized by a common kinetic mechanism [1]. Surprisingly, it was recently found that CAC proceeds via a ping-pong mechanism [47], indicating that only one binding site exists which is alternately exposed to each side of the membrane. The CAC is the only mitochondrial translocator mediating both exchange and uniport. Presumably 're-orientation' or conformational change of the unloaded carrier can be achieved more easily when only binary complexes, and not ternary complexes, are involved in the catalytic cycle. The knowledge of the primary structure of CAC will obviously be needed to answer whether this carrier belongs to the mitochondrial carrier family. However, it should be pointed out that the presence of both the ping-pong and simultaneous mechanisms within the same structural family has been observed before, for example in the family of proteins related to the erythrocyte anion carrier. Apart from this problem, it should be recalled that the well-documented existence of two conformational states of AAC, depending on the side at which the ligands are bound, has been interpreted in terms of re-orientation of the ligand binding site [48]. These findings may be explained by assuming a functional dimer (or tetramer) composed of two monomers with one substrate binding site and one transport channel each [1,4].

By reaction of particular cysteine residues with mercurial reagents, several carriers could be reversibly converted to a functional state different from the various physiological transport modes [1,4,49]. This 'unphysiological' transport mode is characterized by a complete inhibition of the 'physiological' transport functions and by a unidirectional substrate flux with a more or less complete loss of substrate specificity. The new type of activity can be interpreted to indicate the presence of an intrinsic unspecific channel within the mitochondrial carrier proteins, which is normally hidden by appropriate gates. This interpretation may be reconciled with the emerging general concept of the evolution of carrier proteins, starting from simple pores and evolving gradually to more and more complex structures.

7. Expression and site-directed mutagenesis

Despite some previous attemps [50], over-expression of intact mitochondrial carriers in bacteria in the functionally active state has been accomplished only recently. Fiermonte et al. [51] succeeded in expressing in *E. coli* the full-length bovine OGC at a level of about 15 mg/l of culture and the full-length bovine AAC at somewhat lower levels. Both OGC and AAC accumulated as insoluble inclusion bodies in the bacterium. OGC was solubilized with sarcosyl and reconstituted into liposomes. The transport properties of this reconstituted carrier were shown to be the same as those determined for the native OGC in mitochondria. This is the first time that a eukaryotic membrane protein has been over-expressed in *E. coli* and renatured. Since the bovine PiC has also been over-expressed in *E. coli* and refolded into the reconstitutively active form (G. Fiermonte, personal communication), it is possible that also the other mitochondrial carriers may be expressed with the vector used for the expression of the OGC and AAC.

Site-directed mutagenesis studies of AAC and PiC have been performed so far only in yeast, where expression of these proteins was accomplished before this was done in *E. coli*. In the yeast AAC2 essential residues were found to be the three arginines which are located within the transmembrane helices and possibly within the translocation channel, the triplet R252–254 which is located on the matrix side within the third hydrophilic loop and possibly participating in an annular positive field around the channel entrance, and the only lysine (K39) which is located within a transmembrane helix probably involved in substrate binding and translocation [32,52]. Mutation of T43 to C of the yeast PiC resulted in 90% inhibition, suggesting that this residue (corresponding to C42 in the bovine heart PiC or C41 in the rat liver PiC) plays an important role in the P_i transport mechanism [53].

8. Biogenesis and import into mitochondria

Mitochondrial carriers are products of nuclear genes and have to be imported into the mitochondrial inner membrane. AAC, UCP, OGC and the yeast PiC are synthesized without a targeting presequence, whereas the mammalian PiC and CiC carry cleavable presequences of different lengths. The import of AAC, UCP and PiC uses the general mitochondrial import pathway [54–56].

Thus, it requires cytosolic factors, ATP and a membrane potential. Differently from most other mitochondrial proteins, the AAC and the PiC preferentially use the special receptor, MOM72, of the outer membrane as their main import receptor [54,57]. Furthermore, AAC and UCP are probably inserted directly into the inner membrane without first passing through the matrix, i.e. they are imported into the mitochondria through a non-conservative pathway [54,58]. It has also been shown that effective import of AAC and UCP depends on particular regions within their mature sequence [54].

The role of the cleavable presequence of the mammalian PiC has been studied by two groups [56,59]. It appears that the major import information must reside in the mature part of the PiC, as in the case of the other presequence-deficient members of the mitochondrial carrier family. Recently, it has been proposed that the yeast PiC/p32 functions as an import receptor on the outer mitochondrial membrane [60]. However, the submitochondrial localization and the mechanism of import [57], as well as the functional expression in a yeast strain deficient in endogenous PiC [53], characterize the yeast PiC/p32 as a member of the mitochondrial inner membrane carrier family.

9. Conclusions

Although definite progress in the study of the mitochondrial carrier proteins at molecular level has been made in the last few years, we have to admit that the catalytic mechanism of metabolite translocation is not yet known. Several models describing transport mechanisms have been published [4,32], but it is clear that the basic mechanisms of metabolite transport in the case of the mitochondrial carriers or in any other fields of membrane biology will not be understood until the three-dimensional structures of the catalysts become accessible. Crystallization of the mitochondrial carrier proteins may be at hand now that functional over-expression of these transport proteins has been achieved.

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