Surface labeling of membrane-bound ADP/ATP carrier by pyridoxal phosphate

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1. INTRODUCTION

Recently the primary structure of the ADP/ATP carrier from beef heart mitochondria has been established [1]. On this basis, the location of modified amino acid can be determined. There are several reasons for modifying amino acids, e.g., detecting the amino acids which belong to the binding center, elucidating those which are sensitive to the conformational changes between various functional states, or as applied here for elucidating protein topography. In the latter case, particularly surface labeling with either hydrophilic or hydrophobic reagents should mark those amino acids which sit on the corresponding surfaces [2].

Here, we report on the labeling of the ADP/ATP carrier in beef heart mitochondria by the highly hydrophilic lysine reagent PLP. The ADP/ATP carrier contains numerous lysines distributed over the whole sequence, rendering differences in the reactivity more significant. Moreover, with the carrier localized in the intact mitochondrial membrane, PLP can be used to probe from either side of the membrane and differentiate between inside and outside localized lysine residues. The positions of the labeled lysines are established. The consequences for the possible folding of the central section of the ADP/ATP carrier through the membrane will be discussed.

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Abbreviation: PLP, pyridoxal 5-phosphate

2. EXPERIMENTAL

PLP was tritiated according to [3]. Bovine heart mitochondria were loaded with carboxyatractylate [4], and sonic mitochondrial particles [5] prepared as described.

For [^{3}H]PLP incubation, 2 g portions of mitochondria or submitochondrial particles were suspended to 30 mg/ml in 50 mM triethanolamine–HCl, 250 mM sucrose (pH 8.0). The reaction was performed at 20°C for 10 min in the dark at 6 mM final conc. After reduction of the Schiff's base by an equimolar amount of $NaBH_4$ at 4°C, the carboxyatractylate–protein complex was isolated as in [6].

The protein was denatured and after extraction of phospholipids, carboxymethylated and citraconylated. Cleavage by thermolysin, decitraconylation and separation of peptides was done as in [1]. Fractions were pooled in respect to the ^{3}H-radioactivity elution profile. After preparative fingerprinting on cellulose plates the pyridoxyllysine containing peptides were identified by their blue fluorescence under UV lamp, scratched out and eluted. The sample was subjected to manual sequencing [1] and from an aliquot the yield of radioactive peptides was determined.

3. RESULTS

Preliminary experiments with beef heart mitochondria showed that exposure to pyridoxal phosphate did not entail the loss of bound [^{3}H]carboxyatractylate under conditions applied in these

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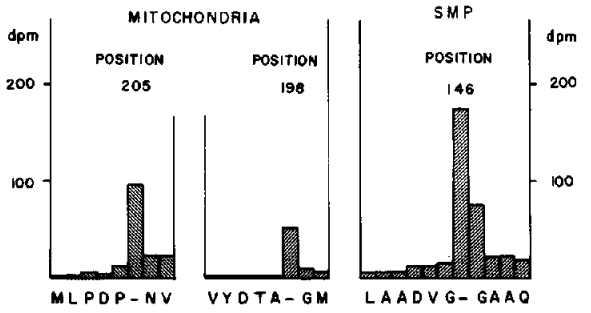

Fig.1. Localization of positions of pyridoxal phosphate incorporation. The purified peptides were sequenced using 4-[4-(dimethylamino)-phenylazo]-phenylisothiocyanate and the resulting thiohydantoin derivatives identified on polyamide sheets [1]. Radioactivity was determined from an aliquot of the butylacetate extract.

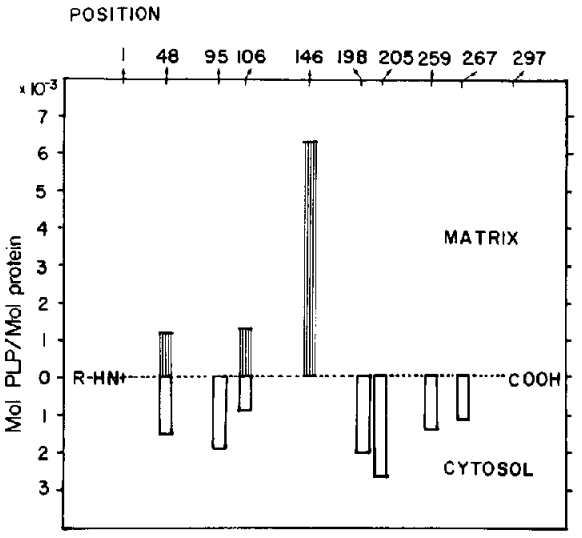

Fig.2. Distribution of lysine residues along the polypeptide chain accessible for pyridoxal phosphate from the cytosolic or matrix side. The incorporation of label into the different lysine positions of thermolysinolytic peptides derived from PLP treatment of beef heart mitochondria (cytosolic side) or submitochondrial particles (matrix side) was determined as demonstrated for 3 examples in fig.1. Because of the broad specificity of thermolysin, for one and the same lysine position several peptides were obtained. The radioactivity of corresponding peptides was summed up and related to the amount of carrier protein before cleavage.

binding studies. Therefore, we conclude that in these experiments the tertiary structure of the ADP/ATP carrier remains intact. For determining accessible groups on the outer ('c'-side) surface of the carrier, PLP was reacted directly with carboxyatractylate loaded mitochondria. To label lysine groups on the inner surface ('m'-side) the loaded mitochondria were first sonicated and then pyridoxal phosphate was reacted on the isolated 'inside-out' submitochondrial particles. After the reaction with [^{3}H]PLP the Schiff's bases were reduced by $NaBH_4$. From these membranes, the ADP/ATP carrier was solubilized and purified by the usual procedures [6]. For localization studies, thermolysinolytic peptides were generated, separated and sequenced according to [1].

The distribution of radioactivity in each degradation step is presented in fig.1 for 3 selected peptides, which derived from mitochondria and submitochondrial particles. There is a good agreement of the maximum of radioactivity with the lysine position, predicted by the known primary structure [1]; however, some tailing is seen in the following steps caused by the poor extractability of the thiazolinone derivatives of *N*-ε-pyridoxyl-lysine [7].

The incorporated amounts of pyridoxal phosphate to the lysines along the amino acid chain (fig.2) are summarized in fig.2. Only 8 out of 23 lysines present in the ADP/ATP carrier are reactive with PLP. Probably the hydrophilic nature of the reagent prevents severely the accessibility to the majority of lysines. These are supposedly either buried in the internal space of the protein or shielded by the membrane. Among the reacting lysines ⅔ rds are accessible from the outer cytosolic side. PLP modification of submitochondrial particles resulted in a predominant modification of Lys 146, but also to a minor extent at Lys 48 and Lys 106. Both these are also labeled in mitochondria. On the other hand, lysine residues at positions 95, 198, 205 and 267 react only in mitochondria.

It should be kept in mind that these results of surface labeling pertain to the 'c'-conformation of the ADP/ATP carrier as established by carboxyatractylate binding. Different lysines may be accessible in the 'm'-conformation which, however, is much more difficult to isolate due to its lability.

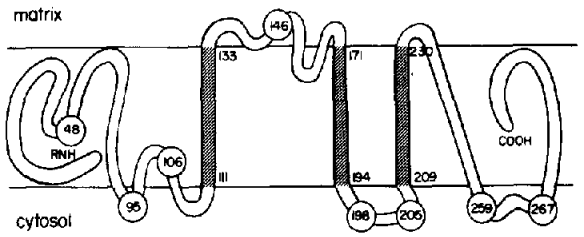

Fig.3. Possible spanning of the polypeptide chain in the membrane as suggested by surface labeling of the carboxyatractylate loaded ADP/ATP carrier with PLP. Three strikingly hydrophobic segments are shown.

4. DISCUSSION

With these data on the surface labeling of lysine by PLP of the membrane-localized ADP/ATP carrier and with our information on the distribution of hydrophobic segments in the sequence of this protein, a first framework is given for spanning the amino acid chain in the membrane. This is shown in fig.3, employing a minimum of further assumptions. It should be noted that this scheme does not depict secondary structure. The labeled lysines are distributed across the membrane according to the reactivity with PLP. The activity both in mitochondria and submitochondrial particles of Lys 48 and Lys 106 is accounted for placing these groups in the center region between both surfaces which admittedly is difficult to rationalize. The hatched membrane traversing segments represent hydrophobic segments of ~20 amino acids. These 3 segments stretching from position 111–133, 171–195 and 209–230 have been designated as candidates for membrane traversing α-helices [1].

Secondary structure assignments by various primary→secondary structure prediction methods are particularly equivocal for membrane proteins. However, according to CD-measurements, the total α-helical content of the ADP/ATP carrier is ~42% (unpublished). We feel that secondary and tertiary structure assignments will be more usefully related to the structures of nucleotide binding proteins rather than to the nearly pure α-helix structure of bacteriorhodopsin.

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