Vol. 141, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 26, 1986 Pages 313-318

MITOCHONDRIAL MALATE DEHYDROGENASE AND ITS PRECURSOR HAVE DIFFERENT CONFORMATIONS

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Received October 20, 1986

Antiserum prepared against the denatured form of mammalian malate dehydrogenase was found to immunoprecipitate the denatured but not the native form of the mature enzyme. In contrast, the antiserum immunoprecipitated the enzyme's precursor, synthesized in a rabbit reticulocyte lysate, either before or after denaturation. The mature form of the enzyme but not the precursor bound to an affinity column of 5'-AMP-Sepharose. These results indicate that the mature and precursor forms of malate dehydrogenase have different conformations.

Mitochondrial malate dehydrogenase from rat is synthesized in the cytoplasm as a precursor about 2,000 daltons larger than the mature monomeric form (1,2). The precursor is imported by isolated mitochondria and processed to the mature size (3,4). The characteristics of its import including initial binding to the mitochondrial outer membrane and dependence on an electrochemical gradient across the inner membrane (3,4) are similar to those of other mitochondrial proteins (5). Recently, rat malate dehydrogenase has been cloned and the 24 amino acid targeting presequence was found to be similar to other imported mitochondrial proteins (6). Further, the presequence of ornithine cabamoyl transferase could compete for the import of this enzyme as well as for malate dehydrogenase (7).

Little is known of the conformation in which proteins are imported into mitochondria. In both Neurospora crassa and yeast it appears that the precursor proteins must cross the mitochondrial membranes in a partially or completely unfolded state (8,9). Here we have examined the conformation of the precursor of malate dehydrogenase by its reactivity with

Abbreviation: SDS, sodium dodecyl sulphate.

0006-291X/86 $1.50

antibodies specific for the denatured form of the enzyme, and by its binding to an affinity column.

MATERIALS AND METHODS

Materials. Protein A-Sepharose CL-4B and 5'-AMP-Sepharose were purchased from Pharmacia Fine Chemicals. [^{35}S]Methionine (~1000 Ci/mmol) was obtained from New England Nuclear.

General Procedures. Synthesis in vitro of malate dehydrogenase precursor in a nuclease treated reticulocyte lysate primed with rat liver free polysomes, and analysis by SDS-10% polyacrylamide gel electrophoresis and fluorography of the dried gel were by methods described previously (1,3,4). Chinese hamster ovary cells were labelled with [^{35}S]methionine and mitochondria isolated as described (10). Malate dehydrogenase activity was measured by following the oxidation of NADH at 340 nm in a solution containing 160 µM NADH, 120 µM oxalate and 0.1M sodium phosphate, pH 7.2.

Immunoprecipitaion. The rabbit antiserum raised against SDS-denatured bovine malate dehydrogenase was that used previously (1,3,4). Malate dehydrogenase was immunoprecipitated from lysates of mitochondria (4 mg protein) isolated from 4.2 x 10^8 [^{35}S]methionine-labelled Chinese hamster ovary cells. For immunoprecipitation without prior denaturation, one-half of the mitochondria were solubilized with a solution containing 0.3 M KCl, 1% (v/v) Triton X-100, 5 mM EDTA and 10 mM Tris-HCl, pH 7.2 at a protein to Triton ratio of 1:3. After 30 min at 0 °C the solution was centrifuged, the supernatant was divided into two and each portion adjusted to 1 ml with the solubilization buffer. For immunoprecipitation after denaturation, the other aliquot of mitochondria was boiled for 5 min in 300 µl of 4% (w/v) SDS and 10 mM mercaptoethanol. After cooling to room temperature, the solution was divided into two equal portions and each was adjusted to 1.0 ml with a solution containing 0.15 M NaCl, 5 mM EDTA, 1% (v/v) Triton X-100 and 50 mM Tris-HCl, pH 7.2. Thirty µl of anti-malate dehydrogenase and preimmune sera were added to each set of immunoreaction mixtures. Similar procedures were used for immunprecipitation of the precursor from a cell-free reticulocyte translation system primed with rat liver free polysomes in the presence of [^{35}S]methionine. Antigen-antibody complexes were recovered with protein A-Sepharose and examined by gel electrophoresis and fluorography.

Affinity chromatography. The binding of malate dehydrogenase and its precursor to an affinity column of 5'-AMP-Sepharose was examined by the method described previously for the isolation of the enzyme (1).

RESULTS

Mature malate dehydrogenase and its precursor have different conformations. The difference in the primary structure of malate dehydrogenase and its precursor indicates that they could also differ in their secondary and tertiary structures. This was shown to be the case by an immunological approach. Previously we had shown that antiserum raised against SDS and mercaptoethanol denatured bovine malate dehydrogenase recognized the mature enzyme, from rat or bovine sources, only if it had been denatured

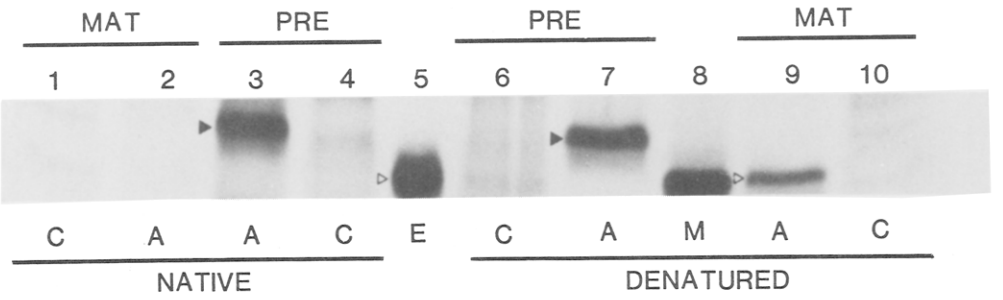

Figure 1. Immunoprecipitation of malate dehydrogenase and its precursor with or without prior denaturation. Mature malate dehydrogenase (MAT) was immunoprecipitated from mitochondria isolated from [^{35}S]methionine-labelled Chinese hamster ovary cells as described in the Materials and Methods. Malate dehydrogenase precursor (PRE) was recovered from 1.75 x 10^6 cpm of the [^{35}S]methionine-labelled post-ribosomal supernatant of a cell-free reticulocyte system primed with rat liver free polysomes. The mitochondria and post-ribosomal supernatant were either denatured by boiling for 5 min in 4% SDS and 10 mM 2-mercaptoethanol before immunoprecipitation or immunoprecipitated directly as indicated. Details of these methods and of the analysis of the immunoprecipitates by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography are described in the Materials and Methods. Antiserum (A) and preimmune serum (C) were used as indicated. Lane 5 is [^{14}C]formaldehyde-labelled bovine malate dehydrogenase (E) and lane 8 is [^{14}C]formaldehyde-labelled molecular mass marker (M) of 34,000 Da (10). ▶, Malate dehydrogenase precursor; ▷, malate dehydrogenase.

before immunoprecipitation (1). This is shown further for the mature enzyme in the results in Fig. 1. The antiserum (A) precipitated the mature enzyme (MAT) from Chinese hamster ovary cells only if it had been denatured (lane 9 cf. lane 2). However, the antibodies immunoprecipitated the precursor (PRE) whether it had been denatured (lane 7) or not (lane 3). Neither precursor nor mature forms of the enzyme were immunoprecipitated with preimmune serum (C). These results indicate that the antibody preparation is specific for a group of antigenic determinants that are present on the denatured mature enzyme, but are not present or exposed when the enzyme is in the native form. Furthermore, the fact that the precursor protein can be immunoprecipitated without prior denaturation indicates that these antigenic determinants are present in the native conformation of the precursor protein.

Malate dehydrogenase precursor does not bind to an affinity column of 5'-AMP-Sepharose. 5'-AMP-Sepharose has been used as an affinity column to purify mitochondrial malate dehydrogenase (1,11,12). The rat liver enzyme binds to the column and can be

Figure 2. Malate dehydrogenase precursor does not bind to a 5'-AMP-Sepharose affinity column. Rat liver free polysomes were translated as described in Fig. 1 and the Materials and Methods. About 7.6 x 10^7 cpm of [^{35}S]methionine-labelled translation products in the post-ribosomal supernatant were applied to a 0.5 ml column of 5'-AMP-Sepharose equilibrated with binding solution containing 4 mM mercaptoethanol, 2 mM EDTA and 20 mM sodium phosphate, pH 7.0. The column was washed with 4 bed volumes of the binding solution, and then eluted after fraction 4 was collected with the same solution containing 40 µM NADH. Fourteen 0.5 ml fractions were collected. The fractions were denatured, immunoprecipitated and analysed as described in the Materials and Methods. Lane 1, [^{14}C]formaldehyde-labelled bovine malate dehydrogenase; lanes 2 - 6, immunoprecipitates from pooled samples of fractions 1 - 3, 4 - 6, 7 - 9, 10 - 12 and 13 - 14, respectively. ▶, Malate dehydrogenase precursor; ▷, malate dehydrogenase.

eluted with 40 µM NADH (1). In contrast, rat liver malate dehydrogenase precursor did not bind to the affinity column being recovered from the flow-through fractions by immunoprecipitation (Fig. 2, lane 2). No precursor was eluted with NADH (Fig. 2, lanes 3-6). Lack of binding of the precursor could have been due to its application to the column in the translation solution in which it was synthesized. However, in a control experiment, mature rat liver enzyme added to the translation solution did bind and 60% and 35% was eluted with 40 µM NADH in fractions corresponding to lanes 4 and 5 respectively. The binding of mature enzyme and the lack of affinity of the precursor for the column indicates that the precursor probably lacks a binding site for NAD^+. However, it can not be excluded that the presequence blocks the site.

DISCUSSION

The immunological results are consistent with a model in which the antibodies react with antigenic determinants that are present on denatured malate dehydrogenase and native precursor but are not present or exposed in the native mature form. Further, the precursor probably lacks the NAD^+-binding site of the mature enzyme. Two inferences can be drawn. First, there is a conformational difference between the mature enzyme and its precursor. Second, there are some conformational similarities between the precursor and SDS-denatured malate dehydrogenase. The simplest explanation is that the precursor is in a partially or

completely unfolded form apparently necessary for import of other precursors into mitochondria (8,9). It should be noted that precursors may be able to adopt a mature conformation. Dihydrofolate reductase does this when attached to the presequence for cytochrome oxidase subunit IV, although in this case the reductase is not a mitochondrial protein (9). In contrast the precursor and mature forms of the ADP/ATP carrier from Neurospora differ in conformation (13). Cytochrome c and its precusor apocyochrome c clearly must differ in conformation. The latter case is of interest in terms of the results reported here. Korb and Neupert were able to raise antibodies specific for apocytochrome c (14). Further antibodies specific for the denatured forms of other proteins, including ribonuclease (15) and lysozyme (16-18), have been previously reported (19). Antibodies against peptide portions of proteins have been found to react with the denatured form of the protein in the case of staphylococcal nuclease (20) and sperm whale myoglobin (21,22).

The simplest model for import is that the precursor is in a form that will permit binding to and transport into mitochondria. This would be the case for a partially or completely unfolded form of malate dehydrogenase precursor. It was suggested that presequences may play a role in preventing premature conformational changes that might interfere with the import process (23). Although there is no direct evidence for this, in the case of malate dehydrogenase a conformational change must occur after import. Removal of the presequence might facilitate this.

ACKNOWLEDGMENTS

We wish to thank Hasmukh Patel for excellent technical assistance and Laurel Aziz for helpful discussions. This research was supported by a grant from the Medical Research Council of Canada.

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