Synthesis in vitro of rat brown adipose tissue 32000 M_r protein

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The synthesis of the mitochondrial inner membrane 32000 M_r protein from rat brown adipose tissue was examined. Polysomes from the tissue were translated in a reticulocyte lysate protein-synthesizing system and newly-synthesized protein isolated with a monospecific antibody against the 32000 M_r protein. The newly-synthesized protein had the same relative molecular mass as the mature protein. It was taken up by mitochondria isolated from Chinese hamster ovary cells into a form resistant to trypsin.

Brown adipose tissue *Mitochondria* *32000 M_r protein*

1. INTRODUCTION

Brown adipose tissue of mammals has the specific function of heat production in the newborn [1], small mammals acclimated to cold [2] and arousal from hibernation [3], and it might also protect from obesity [4]. Heat production in this tissue is under neurohormonal control and most likely involves a specific protein of the mitochondrial inner membrane of brown adipose tissue [5]. This protein, variously termed uncoupling protein, GDP-binding protein, 32000 M_r protein and thermogenin, probably acts by permitting the movement of hydroxide ions out of mitochondria [5]. This would discharge the electrochemical gradient, uncouple mitochondria and produce heat. The specific induction of brown adipose tissue in rats kept in the cold [6] and the large amount of the 32000 M_r protein in it, makes the biosynthesis of this tissue-specific protein of great interest. Here we report on its biosynthesis in a reticulocyte lysate.

2. MATERIALS AND METHODS

[^{35}S]Methionine and [^{14}C]formaldehyde, each at the highest specific radioactivity available, were obtained from New England Nuclear Canada (Lachine, Quebec). Protein A–Sepharose CL-4B was purchased from Pharmacia Fine Chemicals (Uppsala).

The 32000 M_r protein was purified by hydroxylapatite chromatography [7] from mitochondria [8] of interscapular brown adipose tissue of cold-acclimated Sprague-Dawley rats. The purified protein in 20 mM Na_2SO_4, 0.15 mM EDTA, 20 mM 4-morpholinepropane-sulphonate (pH 6.7) and 3–5% (w/v) Triton X-100 was used directly for antibody induction. About 500 μg protein was injected intramuscularly and subcutaneously into rabbits every 2 weeks, first with complete Freund's adjuvant and then with incomplete Freund's adjuvant. The rabbits were bled 7 days after the third and subsequent injections. The activities of antisera were assayed by protein A–Sepharose precipitation of antibody–antigen complexes [9] using [^{14}C]formaldehyde-labelled [10] 32000 M_r protein.

Free polysomes were prepared from interscapular brown adipose tissue of cold-acclimated rats or from rat liver essentially as in [11]. Protein synthesis was in a polysome-primed nuclease-treated reticulocyte lysate [12]. Newly-synthesized protein was isolated from the ribosomal supernatant by precipitation with antiserum and protein A–Sepharose [9]. Precipitates were examined by sodium dodecyl sulfate–

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polyacrylamide slab gel electrophoresis and fluorography [13].

Uptake of newly-synthesized protein was examined by incubating about 4.4×10^7 cpm of the ribosomal supernatant with about 600 μg Chinese hamster ovary cell mitochondria prepared as in [13]. After incubation of the 0.4 ml mixture for 45 min at 30°C, mitochondria were either recovered directly, or the incubation mixture was treated with trypsin (6 μg/100 μg mitochondrial protein) at 0°C for 30 min, protease inhibitors added and then mitochondria recovered. In each case mitochondria were washed and both mitochondria and supernatant fractions were analyzed by immunoprecipitation followed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

3. RESULTS

3.1. *Purification of 32000* M_r *protein and antibody specificity*

Purification by hydroxylapatite chromatography proved effective in obtaining pure 32000 M_r protein. Only one electrophoretic band was observed with Coomassie brilliant blue staining (not shown) or with [^{14}C]formaldehyde-labelled purified protein (fig.1, lane 2). This protein was considered identical to that described by others on the basis of its size, method of purification, and specific presence in large amounts in brown adipose tissue.

Antibodies against the 32000 M_r protein immunoprecipitated the [^{14}C]-labelled purified protein (fig.1, lane 3). No precipitation was observed with preimmune serum (not shown). The antibodies appeared monospecific on the basis of 'Western' blot analysis of rat brown adipose tissue and liver mitochondrial proteins [14] (not shown) and from the biosynthetic result described below.

3.2. *Product of cell-free protein synthesis primed with rat brown adipose tissue polysomes*

The newly-synthesized form of the 32000 M_r protein was isolated with antiserum from a brown adipose tissue polysome-primed reticulocyte lysate translation mixture. The newly-synthesized protein had the same electrophoretic mobility as the mature protein (fig.2A, lane 3 cf. lane 2). Identity of the newly-synthesized protein with the 32000 M_r

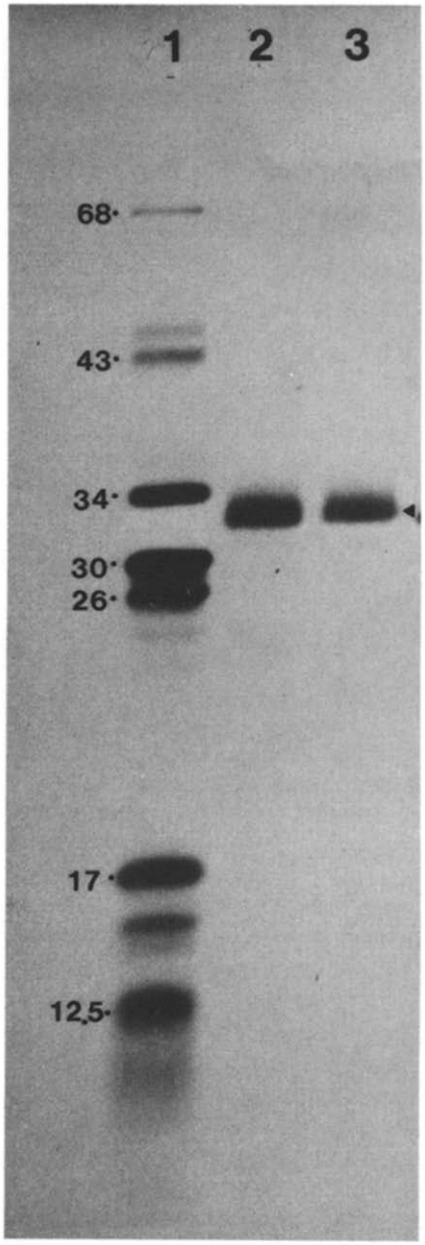


Fig.1. Sodium dodecyl sulfate–12.5% polyacrylamide slab gel electrophoresis and fluorography of 32000 M_r protein and its immunoprecipitation with antiserum. The purified 32000 M_r protein was labelled with [^{14}C]formaldehyde (lane 2) and immunoprecipitated (lane 3). Lane 1 shows [^{14}C]formaldehyde-labelled relative molecular mass markers. The numbers on the left denote molecular masses ($\times 10^{-3}$).

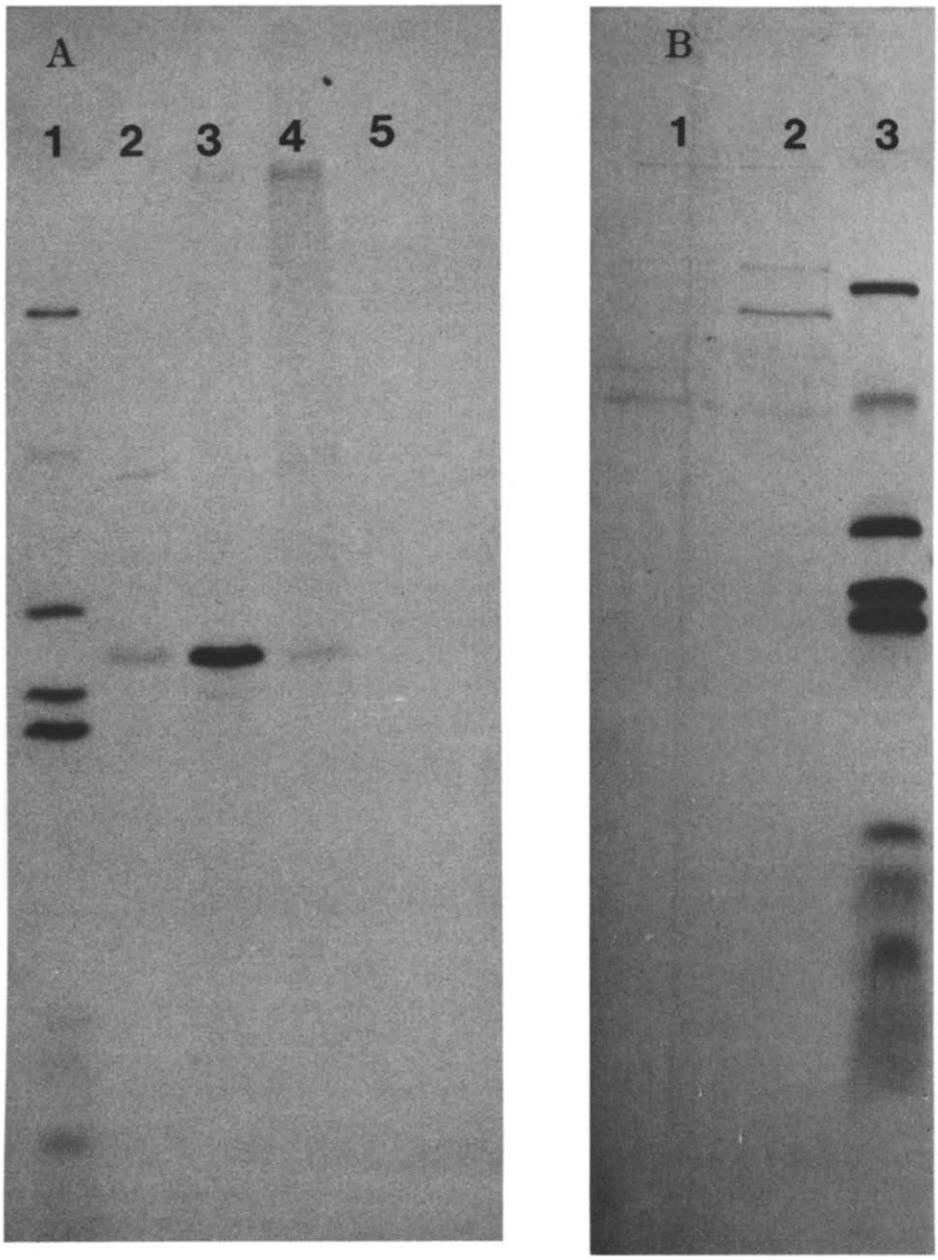
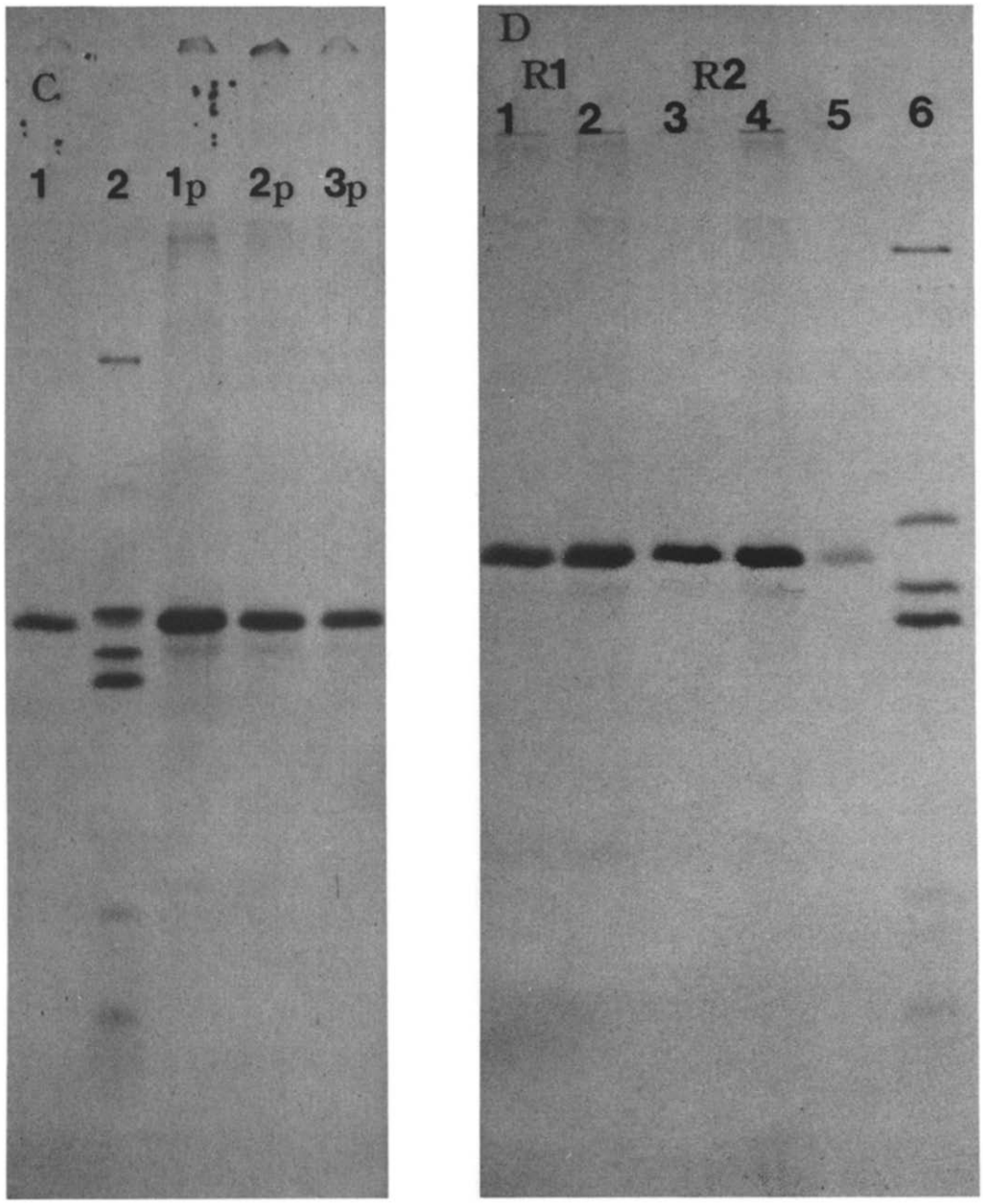


Fig.2. Sodium dodecyl sulfate–12.5% polyacrylamide slab gel electrophoresis of immunoprecipitates from rat polysome-primed reticulocyte lysates. Polysomes from rat brown adipose tissue or liver were incubated at 20 A_{260} unit/ml with [^{35}S]methionine at 0.75 mCi/ml in a nuclease-treated reticulocyte lysate for 30 or 60 min at 30°C. Each immunoprecipitation was of 40–60 μl of the translation mixture and with 20 μl of the antiserum or preimmune serum. (A) Comparison of the 32000 M_r protein (lane 2) with immunoprecipitates from translation products of brown adipose tissue polysomes with antiserum (lanes 3,4) or preimmune serum (lane 5). In lane 4, 20 μg of non-radioactive 32000 M_r protein was added before immunoprecipitation. Lane 1: relative molecular mass markers. (B) Immunoprecipitation of translation products of rat liver polysomes with antiserum (lane 2) and preimmune serum (lane 1). Lane 3: relative molecular mass markers.



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protein was indicated by competition for immunoprecipitation by non-radioactive 32000 M_r protein (lane 4), and lack of precipitation by preimmune serum (lane 5) or from a rat liver polysome-primed reticulocyte lysate (fig.2B). These results show that the newly-synthesized protein is unique to brown adipose tissue, and most likely is the nascent form of the 32000 M_r protein.

The finding of the same relative molecular mass for newly-synthesized and mature protein was not dependent on polysome preparation (fig.2C) or on antiserum preparation (fig.2D). More newly-synthesized protein was recovered by antiserum from the second bleeds (lane 2,4) compared to their respective first bleeds (lanes 1,3). It should be noted that in all cases a protein of about 29000 M_r was also observed with the newly-synthesized

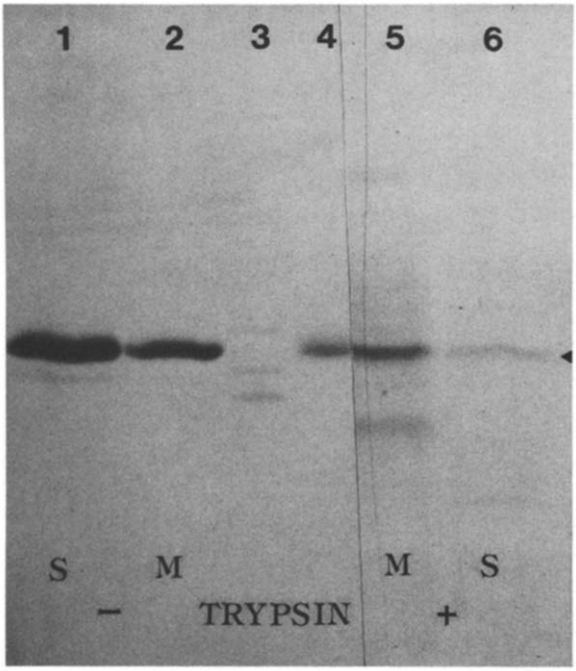

Fig.3. Uptake of newly-synthesized 32000 M_r protein by isolated mitochondria of Chinese hamster ovary cells. The experimental details are given in section 2. Lanes 1,2, immunoprecipitates from supernatant (S) and mitochondrial (M) fractions; lane 3, relative molecular mass markers; lane 4, [^{14}C]formaldehyde-labelled 32000 M_r protein; lanes 5,6, immunoprecipitates from mitochondrial and supernatant fractions after trypsin treatment of the translation mixture. Lanes 5,6 are from a different exposure of the same gel as the other lanes.

32000 M_r protein. This protein was <5% of the 32000 M_r protein. Its origin is not known.

3.3. *Uptake of newly-synthesized 32000* M_r *protein by isolated mitochondria*

The newly-synthesized 32000 M_r protein was found in the mitochondrial fraction after incubation of the translation mixture with mitochondria isolated from Chinese hamster ovary cells (fig.3, lane 2). Trypsin treatment hydrolyzed virtually all of the newly-synthesized 32000 M_r protein in the supernatant (lane 6) but not that in the mitochondrial fraction (lane 5). Densitometric scans of the lanes (1,2,5,6) in fig.3 showed that the ratio of the protein in mitochondrial to supernatant fractions was 0.35 before and 4.4 after trypsin treatment. About 45% of the 32000 M_r protein remained in the mitochondrial fraction after trypsin treatment. Protease resistance of the 32000 M_r protein in the mitochondrial fraction indicates its import into mitochondria, and perhaps into its final location, the mitochondrial inner membrane.

4. DISCUSSION

Most of the mitochondrial proteins synthesized on cytoplasmic ribosomes are made in precursor form larger than the mature protein [15,16]. One exception is the adenine nucleotide carrier of *Neurospora crassa* [17]. We find that this protein is synthesized in precursor form of higher relative molecular mass in reticulocyte lysates primed with rat liver RNA [18], although no precursor of larger relative molecular mass was observed in whole cell experiments by others [19]. The finding that the 32000 M_r protein is not made in precursor form, within the limit of relative molecular mass analysis by gel electrophoresis [13], shows the diversity amongst imported mitochondrial proteins. This variation strongly indicates that the function of the N-terminal extension [20–22] of the precursor proteins, when it exists, is not similar to that of the 'signal' peptide of excreted proteins [23].

Although uptake of newly-synthesized mitochondrial proteins by isolated mitochondria has been extensively documented in yeast [24,26] and *Neurospora* [27,28], uptake of ornithine transcarbamoylase only has been thoroughly shown in the mammalian case [29]. The results presented here extend our observation with the

precursor of malate dehydrogenase [18] and indicate that uptake in vitro can probably be demonstrated in most cases. Further, the fact that uptake of rat brown adipose tissue 32000 M_r protein was observed with Chinese hamster ovary cell mitochondria indicates that the as yet unidentified receptors for importing mitochondrial proteins [30] are not specific for mammalian species or tissue.

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