Differences in thermal stability of the fetal and adult brain *myo*-inositol-1-phosphate synthase

Probable involvement of NAD

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L-*myo*-inositol-1-phosphate synthase (EC 5.5.1.4) from mammalian fetal and adult brain differ considerably with respect to their stability towards different temperatures between 25–65°C. This property has been found to be associated with the presence of the synthase co-factor, NAD, bound to the enzyme protein. The lower thermal stability of the fetal enzyme increases in presence of added NAD (0.8 mM) whereas the higher thermal stability of the adult brain enzyme declines when NAD is specifically removed from the enzyme.

Inositol synthase *Thermal stability* *Bound NAD* *Mammalian brain* *Human fetus*

1. INTRODUCTION

The NAD-dependent *myo*-inositol-1-phosphate synthase (EC 5.5.1.4) catalyzes the conversion of D-glucose-6-phosphate to L-*myo*-inositol-1-phosphate, which ultimately generates free *myo*-inositol by a specific *myo*-inositol-1-phosphate phosphatase (EC 3.1.3.25). Although complete purification and characterization of the synthase has so far been made for the enzymes obtained from the testis [1,2] or the yeast, *Saccharomyces cerevisiae* [3], inositol synthase in mammalian brain has been worked out only partially [1,2]. In this organ, inositol biosynthesis has been postulated [4] to be correlated with the fructose 1,6-bisphosphatase activity by way of 'rescue synthesis', for cell survival. Keeping this model in view, we have undertaken an investigation on inositol biosynthesis in fetal and adult mammalian brain. Considerable difference in thermal stability of inositol synthase from the two systems was noted. We here deal with this property of the enzyme and its relationship with bound NAD.

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2. MATERIALS AND METHODS

2.1. *Materials*

Albino rats were obtained from local suppliers and then acclimatized for at least 7 days before use. Human fetal brain samples of different gestation periods were obtained following the recommendations of the Ethical subcommittee as described in [5].

D-glucose-6-phosphate (disodium salt), β-NAD, Tris (hydroxymethyl) aminomethane, streptomycin sulfate were from Sigma (St Louis MO). 2-Mercaptoethanol and sodium periodate were from BDH. Sodium sulfite was from May and Baker, and all other chemicals were of the analytical reagent grade available.

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2.2. *Methods: partial purification of the brain enzyme*

All operations were carried out at 0–4°C. Human fetal brain tissue was homogenized in a double volume of chilled 0.154 M KCl containing 0.2 mM 2-mercaptoethanol, and was centrifuged at 8500 × *g* for 30 min in a refrigerated Janetzki K-24 centrifuge. The 8500 × *g* supernatant was treated with streptomycin sulfate to a final concentration of 2% (w/v), kept chilled for 15 min and the pellet spun down. A 30–55% ammonium sulfate fraction obtained from this supernatant, dissolved and dialyzed against the homogenizing solution was layered onto a column of Sephadex G-200 suspended in 50 mM Tris–acetate (pH 7.5) with 0.2 mM mercaptoethanol and 0.1 M KCl. The column was washed with the same buffer and the enzyme eluted following the effluent.

Rat adult brain inositol synthase was prepared essentially following the above procedure, but for one additional step. The 8500 × *g* supernatant was subjected to a heat treatment at 60°C for 2–3 min, chilled and the precipitated protein removed by centrifugation prior to streptomycin sulfate precipitation.

The 30–40% ammonium sulfate fraction was taken for filtration through Sephadex G-200.

The partially purified inositol synthase thus obtained from the two sources showed 6–8 protein bands on polyacrylamide gel electrophoresis under non-dissociating conditions [6]. In both cases, the second band has been found to be associated with the enzyme activity. Details of the purification will be published elsewhere.

Myo-inositol-1-phosphate synthase was assayed by the periodate oxidation method [7] as well as by *myo*-inositol-1-phosphate phosphatase catalysis [8], as detailed in [9]. Protein was estimated as in [10].

3. RESULTS AND DISCUSSION

During our attempts for purification of the human fetal brain synthase, it was revealed that heating the fetal brain preparation for 2–3 min at 60°C, as for the adult rat brain enzyme [1], consistently inactivated the fetal brain synthase. To ascertain whether this specific inactivation of the fetal brain enzyme is, in fact, related to the thermal stability of the two enzymes in question, the crude enzyme preparations (0–80% saturated ammonium sulfate fraction of the 8500 × *g* supernatant) from the two sources were preincubated at temperatures between 25 and 65°C for 5 min prior to the actual enzyme incubation at 37°C for the desired period. It was revealed from such experiments that the adult brain enzyme shows a preincubation temperature maximum at 45°C while that for the fetal brain enzyme is at 35°C. Following this observation, and keeping in view the probable thermolability of the fetal enzyme, inositol synthase from the two sources was purified as described in section 2.2 and the above experiments were performed with the Sephadex G-200 fraction of the enzyme(s). The results of these experiments with the purified enzymes were identical to those of the corresponding crude enzyme preparations (fig.1A). The difference in the thermal stability of the two enzymes was thus evident. However, in

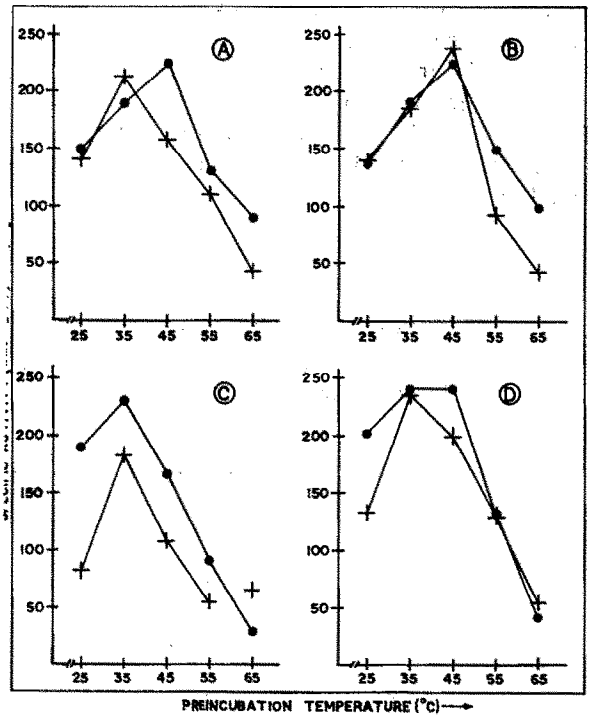

Fig.1. *Myo*-inositol-1-phosphate synthase activity of adult (●—●) and fetal (+—+) brain as a function of preincubation temperature under different conditions. (A) Native enzyme(s) with no addition. (B) Native enzyme(s) with added NAD (0.8 mM). (C) Charcoal-treated enzyme(s) with no addition. (D) Charcoal-treated enzyme(s) with added NAD (0.8 mM).

another set of experiments in which the preincubation of either the fetal or adult brain enzyme was done together with all the components of the assay mixture, the preincubation temperature profile for the two enzymes was found to be indistinguishable; i.e., both the enzymes showed maximum activity at a preincubation temperature of 45°C. This clearly indicated that some component in the assay system either alone or together with others confers thermal stability to the fetal brain enzyme making it qualitatively similar to the adult brain enzyme.

In order to find out the 'responsible component(s)' conferring thermal stability to the fetal brain synthase, preincubation of the enzyme at different temperatures was done with each of the different components of the assay mixture added separately. Results from such experiments revealed that either of the two components of the assay mixture, namely NAD (0.8 mM) or mercaptoethanol (ME; 5 mM), was effective (NAD > ME) in shifting the preincubation temperature maximum of the fetal brain synthase towards 45°C; i.e., the preincubation temperature maximum of the adult brain enzyme. NAD alone is almost as effective as the mixture of all the assay components together (fig.1B).

Since inositol synthase from various sources has been reported to contain bound NAD [11], the probable presence of enzyme-bound NAD as an explanation for the difference in thermal stability between the two enzymes was considered. Following treatment with activated charcoal, a procedure known to remove enzyme-bound NAD [11], the preincubation temperature maximum for the adult rat brain synthase dropped to 35°C from the usual 45°C, while that for the human fetal brain enzyme remained unaltered at 35°C (fig.1C). However, in contrast to the untreated fetal enzyme, both the adult and fetal charcoal-treated enzymes show variable responses in regaining sharp temperature maxima at 45°C when NAD (0.8 mM) was added to this system (fig.1D; cf. fig.1B); nevertheless, increased activity of the two enzymes was evident at least up to 55°C.

Further evidence in favour of the assumption that the two enzymes differ substantially in their bound NAD content came from experiments described in table 1. The adult rat brain untreated enzyme retains about 70% of the activity in the absence of added NAD, whereas only about 43% of the enzyme activity was retained by the fetal brain synthase under identical conditions. On charcoal treatment, however, both the enzymes

Table 1

Alterations in brain synthase activity on removal of bound NAD and its recovery

Enzyme source	Conditions	Spec. act. (nmol inositol 1-P formed. h^{-1}. mg protein^{-1})	Enzyme activity (%)[a]	Drop-in activity (%)
Adult brain (rat)	Untreated			
	− NAD	150	69.7	30.3
	+ NAD	215	100	0
	Charcoal-treated			
	− NAD	32.2	16.5	83.5
	+ NAD	193	89.7	10.3
Fetal brain (human)	Untreated			
	− NAD	86	43	57
	+ NAD	200	100	0
	Charcoal-treated			
	− NAD	27	13.5	86.5
	+ NAD	184	92	8.0

[a] 100% Activity corresponds to the activity of the untreated enzyme(s) in the presence of the optimal concentration of NAD (0.8 mM)

behave similarly in the sense that each loses between 83–87% of activity in the absence of NAD, while about 90–92% activity is regained with NAD (0.8 mM) added to the system.

Our experiments suggest that the thermal stability of the brain inositol synthase is associated with the NAD bound to the enzyme. Although in addition to NAD, the influence of other proteins present in the partially purified enzyme preparation in modulating the enzyme property may not be totally ruled out at this stage, the predominant participation of this co-factor is obvious.

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