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Human placental ATP diphosphohydrolase is a highly *N*-glycosylated plasma membrane enzyme

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Abstract

Human placental ATP diphosphohydrolase (ATP-DPH), has been previously characterized as an azide-sensitive, Ca^{2+}- or Mg^{2+}-dependent triphospho- and diphosphonucleosidase which migrates as an 82 kDa protein band on SDS-PAGE (Christoforidis, S. et al. (1995) Eur. J. Biochem. 234, 66–74). In this paper we have studied the subcellular localization of placental ATP-DPH by differential centrifugation and flotation experiments. Using specific enzymatic markers it was found that ATP-DPH is localized on plasma membrane. ATP-DPH was found to be a highly *N*-glycosylated protein which is a common post-translational modification of plasma membrane proteins. Extensive incubation of the native pure enzyme with *N*-glycosidase F resulted in the elimination of the 82 kDa form and the concurrent formation of a deglycosylated product of 57.5 kDa and four other intermediate products, indicating the presence of at least five *N*-glycosylation sites within the ATP-DPH molecule. The partially deglycosylated sample retained its activity in solution and in native gel electrophoresis and activity staining.

Keywords: ATP diphosphohydrolase; Apyrase; ADPase; Deglycosylation; Subcellular localization; Human placenta

1. Introduction

ATP diphosphohydrolase (ATP-DPH, apyrase, ATPDase, EC 3.6.1.5) hydrolyses triphospho- and diphosphonucleosides to monophosphonucleosides at a similar rate in the presence of Ca^{2+} or Mg^{2+}.

Mammalian ATP-DPHs have been found to be glycoproteins [1–3] mainly localized in plasma membranes [4–7]. In addition to the plasma membrane, the pancreatic enzyme was also detected in Golgi transsacules, and on zymogen granules [8,9]. ATP-DPH from bovine aorta (endothelial and smooth muscle cells) and trachea (non-vascular smooth muscle cells) was characterized as an ecto-enzyme [6,7].

In human placenta a tri- and dinucleotidase activity was first reported by Cerletti et al. [10]. Later, an ADPase activity was detected [11,12] which was initially attributed to alkaline phosphatase [11]. We have previously reported the identification of an ATP-DPH activity sensitive to azide inhibition in human placenta and proposed that this activity is responsible for the hydrolysis of ADP in this tissue [13]. Kettlun et al. [14] described some properties of human placental ATP-DPH. The enzyme was found to be a 64 kDa glycoprotein, activated by a soluble protein, and inhibited by another protein loosely bound to membranes. However, they did not detect sensitivity to azide. Recently, we have purified ATP-DPH from human placenta and characterized the purified enzyme as a glycoprotein with a molecular mass of 82 kDa on SDS-PAGE [1]. However, the functional significance of the carbohydrates on the activity of this enzyme is not yet known. The enzymatic activity is dependent on divalent cations Ca^{2+} or Mg^{2+}, and exhibits broad substrate specificity hydrolysing all triphospho- and diphosphonucleosides with a K_m for ATP or ADP in the μmolar concentration. The optimum pH ranges between 7.5 and 8.0. NaN_3 and NaF have been found to inhibit the enzymatic activity.

Glycosylation of proteins has been proposed to play a key role in the stabilization of protein conformation, protection of proteins from proteases, modulation of protein functions, mediation of cell-matrix and cell–cell interactions and control of half-life of proteins [15]. Some enzymes lose their activity upon deglycosylation, whereas others retain it [15]. Based on the above studies, it appears that the contribution of glycosylation on catalytic activity depends on the nature of the particular enzyme.

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In the present study we have attempted to detect the subcellular localization of human placental ATP-DPH by conventional fractionation and flotation techniques. Furthermore, we report the type of glycosylation, and the role of the carbohydrates on the activity and the molecular weight of human placental ATP-DPH.

2. Materials and methods

2.1. Materials

Human term placenta was obtained after normal delivery, placed on ice and immediately processed. *N*-Glycosidase F (25 000 U/mg) and Triton X-100 were obtained from Boehringer Mannheim. Potato apyrase (625 U/mg) was purchased from Sigma Chemical Company. Other chemicals were of the best available commercial grade.

2.2. Enzymatic assays

2.2.1. ATP diphosphohydrolase

ATP diphosphohydrolase was assayed according to Ames [16] at 30°C in 50 mM Tris-HCl, 1 mM $MgCl_2$, 1 mM ADP (pH 7.5) (800 μl). The reaction was started by the addition of enzyme and allowed to proceed for 10 min. Aliquots (300 μl) were assayed for inorganic phosphate at zero time and 10 min. The phosphate release was within the linear range, and the enzymatic activity was expressed in units of μmol P_i/min.

2.2.2. 5′-Nucleotidase

5′-Nucleotidase (5′-Nase) activity was assayed as indicated for ATP-DPH except that 1 mM AMP was used as substrate.

2.2.3. NADPH-cytochrome-c reductase

NADPH-cytochrome-*c* reductase was assayed at room temperature as described [17] using 50 mM Tris-HCl (pH 7.5), 0.1 mM cytochrome *c*, 0.6 mM KCN and 0.1 mM NADPH (1 ml). Reduction of cytochrome *c* was monitored at 550 nm as a function of time.

2.2.4. Cytochrome-c oxidase

Cytochrome-*c* oxidase was assayed in 50 mM Tris-HCl (pH 7.5) and 0.05 mM reduced cytochrome *c* (1 ml) [18]. Reduced cytochrome *c* was prepared by dithionite treatment of oxidized cytochrome *c*. Excess of dithionite was removed by gel filtration on a G-25 column. Oxidation was followed by monitoring the decrease of absorbance at 550 nm as a function of time.

2.3. Tissue fractionation

Tissue fractionation was performed according to Fleischer and Kervina [19] with some modifications. Briefly, 50 g fresh human term placenta was macerated with razor blades. Five volumes of 10 mM HEPES/NaOH, 0.25 M sucrose, 1 mM PMSF (phenylmethylsulfonyl fluoride) (pH 7.5), were added and the suspension was homogenized in a Potter-Elvehjem homogenizer using a Heidolph stroker at a low speed. The homogenate was filtered through four layers of cheese-cloth and the filtrate (Homogenate) was centrifuged at 3500 RPM for 10 min in a SS-34 rotor ($1000 \times g$) in a Sorvall RC-2B centrifuge at 4°C.

The nuclear pellet (Ppte $1000 \times g$, Nu) was saved and the supernatant was recentrifuged as above. The pellet was discarded and the supernatant was filtered through four double layers of cheesecloth (S_1) and centrifuged at 10 000 RPM for 10 min in a SS-34 rotor ($10\,000 \times g$). The mitochondrial pellet (Ppte $10\,000 \times g$, Mt) was saved and the supernatant was centrifuged as above. The pellet obtained was discarded and the resulting supernatant (S_2) was further centrifuged at $100\,000 \times g$, for 60 min in a 70 Ti rotor in a Beckman ultracentrifuge, model L8-70. The high speed pellet, termed microsomal fraction (Ppte $100\,000 \times g$, Mc) was saved and the supernatant was centrifuged as above. The resulting pellet was discarded, while the postmicrosomal supernatant (S_3) was saved.

2.4. Purification of plasma membranes (flotation experiment)

Purification of plasma membranes was performed according to Aronson and Touster [20], with minor modifications. Briefly, the mitochondrial fraction (Mt) and the microsomal pellet (Mc) were suspended separately in 5.8 ml of 1.665 M sucrose in 10 mM HEPES/NaOH, 0.1 mM PMSF (pH 8.0). Each suspension was placed in the bottom of a 18-ml tube and overlayered by two layers of sucrose: 8.7 ml 0.993 M sucrose in the above buffer was placed first, and on top 2.5 ml 0.25 M sucrose in the same buffer was layered. Tubes (17 ml total volume each) were centrifuged at $100\,000 \times g$, for 16 h in a SW 27 rotor at 4°C. At the end of the run, the tubes were fractionated from top to bottom into five fractions (3.0, 1.5, 6.0, 3.0 and 3.5 ml) for the microsomal pellet and into 6 fractions (2.5, 2.0, 5.5, 3.5, 3.5 ml and pellet) in the case of the mitochondrial sample.

2.5. Purification of human placental ATP-DPH

Human placental ATP-DPH was purified according to Christoforidis et al. [1]. The specific activity of the final preparation was 20 μmol/min per mg, and the activity was free of alkaline phosphatase.

2.6. Protein determination

Protein was determined by the Lowry method [21]. Protein concentration in samples containing detergents was measured by the Bio-Rad assay according to Bradford [22]. Bovine serum albumin was used as a standard.

2.7. Gel electrophoresis

SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli [23] and proteins were visualized by silver staining [24].

PAGE under native conditions was performed as previously described [1] and gels were stained for activity as follows. The gels were washed with 25 mM HEPES/NaOH, 1 mM $MgCl_2$ (pH 7.5), 2 × 10 min and then incubated in the same medium containing 2 mM ADP or ATP and 2 mM $Pb(NO_3)_2$ at 30°C. The reaction was stopped after 4 h by washing with distilled water and the precipitated $Pb_3(PO_4)_2$ was stained black with 1% ammonium sulfide [25].

2.8. Treatment of ATP-DPH with N-glycosidase F

Pure ATP-DPH was treated with *N*-glycosidase F according to the manufacturer's instructions. Briefly, 2.5 μg placental ATP-DPH (as stated) was incubated with 2.34 units *N*-glycosidase F (unless stated otherwise) in 20 mM HEPES/NaOH, 10 mM EDTA (pH 7.5), in a total volume of 20 μl, at 37°C for 12 h. Where indicated, incubation was continued with 2 additional portions of *N*-glycosidase F, at 12 and 24 h. Sodium azide and phosphate buffer which are present in the commercial preparation of *N*-glycosidase F, interfere with the enzymatic assay of ATP-DPH. They were removed by extensive dialysis of the incubation mixture in 2 × 2 l of buffer containing 50 mM Tris-HCl, 1 mM $MgCl_2$ (pH 8.0). Prior to dialysis, 780 μl of the above buffer were added to the sample. When ATP-DPH activity was tested by native gel electrophoresis and activity staining, dialysis was omitted.

Denaturation prior to deglycosylation was performed by boiling the enzyme for 5 min in the presence of 0.1% SDS. Subsequently, Triton X-100 was added to a final concentration of 0.5% to prevent denaturation of *N*-glycosidase F by SDS.

3. Results

3.1. Subcellular localization of ATP-DPH

We have attempted to determine the subcellular localization of placental ATP-DPH by differential centrifugation and flotation experiments. The tissue was fractionated according to the procedure of Fleischer and Kervina [19] and the activity of ATP-DPH in each subcellular fraction was followed in parallel with typical marker enzymes. The distribution of ATP-DPH was found to be identical to that of 5′-nucleotidase, a marker of plasma membrane [26], suggesting that ATP-DPH is localized on the cell membrane (Table 1). A loss of ATP-DPH and 5′-nucleotidase activities was observed when S_1 and S_2 fractions were centrifuged, with no parallel loss in protein. This result might be due to loss of lipids that stabilize these plasma membrane enzymes. Indeed, on addition of phosphatidylcholine, ATP-DPH activity was found to be increased (unpublished observations).

To obtain a pure population of plasma membranes and study further the localization of ATP-DPH, the microsomal pellet was fractionated by flotation as described in Section 2. ATP-DPH activity was followed in parallel with enzyme marker activities. As shown in Fig. 1, fraction 2 contains 5′-Nase and ATP-DPH activities at a similar yield, while it is free of mitochondria and microsomes as judged by the absence of the corresponding enzymatic markers. The distribution of ATP-DPH and 5′-Nase in the other fractions is also similar. An identical pattern of 5′-Nase and ATP-DPH distribution was obtained when the mitochondrial fraction was fractionated by flotation (data not shown). These results support the notion that placental ATP-DPH is a plasma membrane enzyme.

To compare ATP-DPH from plasma membranes with the purified enzyme, native gel electrophoresis and activity staining were used. Purified plasma membrane fraction (Fig. 2, panel ADP, lane 1) contained an activity with the

Table 1
Localization of ATP-DPH activity in human placental subcellular fractions

Fraction	Activity [a] %				
	ATP-DPH	5′-Nucleotidase	NADPH cytochrome-*c* reductase	Cytochrome-*c* oxidase	Protein [b] (mg)
Homogenate	100	100	100	100	525
Nu [c]	4	1	6	10	66
S_1	93	94	92	70	403
Mt	23	24	39	81	66
S_2	43	42	60	0	359
Mc	25	27	26	1	60
S_3	3	5	35	0	365

[a] Enzymatic activities were determined as described in Section 2.
[b] Protein was measured by the Lowry method [21].
[c] Abbreviations of the subcellular fractions are defined in Section 2.

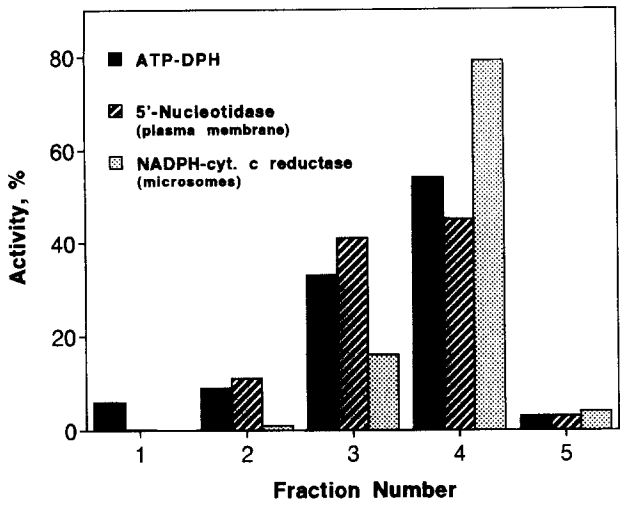

Fig. 1. Isolation of plasma membranes by flotation. Plasma membranes from microsomal pellet were purified by flotation as described in Section 2. The purity of the preparation was assessed by determination of marker enzymes (5'-nucleotidase, NADPH-cytochrome-*c* reductase and cytochrome-*c* oxidase). Enzyme activities are expressed as percentage of the initial units. Note that fraction 2 is enriched in 5'-nucleotidase and ATP-DPH. Cytochrome-*c* oxidase activity of the microsomal fraction was negligible (see Table 1) and is not shown.

same migration pattern as the pure enzyme (Fig. 2, panel ADP, lane 2). Both bands were diminished by NaN_3, a known inhibitor of ATP-DPH [1,2,4,5,7,8,13,27] (Fig. 2, panel ADP + NaN_3, lanes 1, 2), which further supports the presence of placental ATP-DPH in plasma membranes. The upper band of ADP degrading activity detected in the plasma membrane fraction was inhibited by L-phenylalanine, an inhibitor of alkaline phosphatase [28] (Fig. 2, panels ADP and ADP + L-Phe, lane 1), suggesting that this activity is due to alkaline phosphatase. The existence of

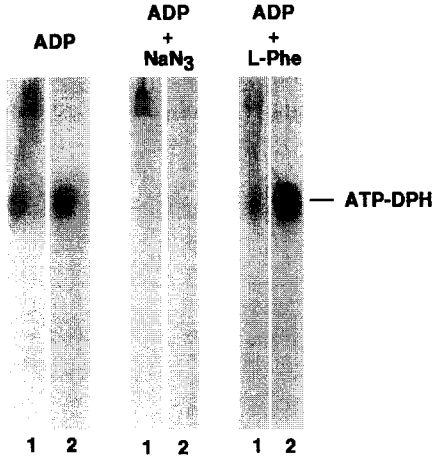

Fig. 2. Detection of ATP-DPH by native PAGE and activity staining in the plasma membrane fraction. Purified plasma membranes, fraction 2 from the flotation experiment (10 μg, lane 1) and pure enzyme (0.3 μg, lane 2) were subjected to native PAGE and stained for activity using 1 mM ADP, or ADP+20 mM NaN_3, or ADP+40 mM L-phenylalanine. Note the activity of the plasma membrane fraction which comigrates with pure ATP-DPH and is inhibited by NaN_3.

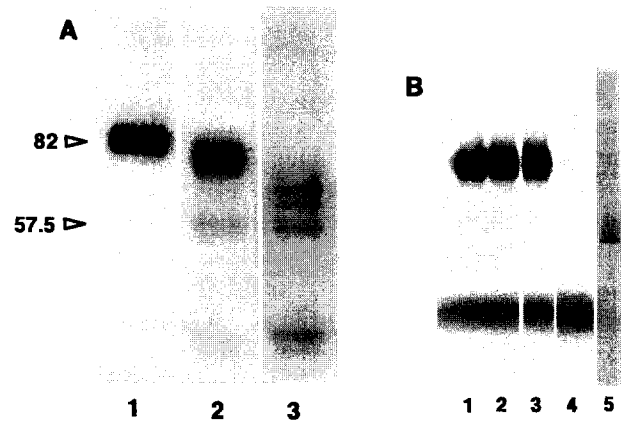

Fig. 3. Effect of *N*-glycosidase F on the molecular weight and on activity of native ATP-DPH. (A) Purified ATP-DPH (2.5 μg, lane 1), pure native ATP-DPH (2.5 μg) treated with 2.34 U *N*-glycosidase F for 12 h (lane 2), and pure native ATP-DPH (2.5 μg) treated for 36 h with additions of 3.9 U *N*-glycosidase F at 0, 12 and 24 h (lane 3) were subjected to 10% SDS-PAGE and protein bands were detected by silver staining. Note the elimination of 82 kDa form after extensive deglycosylation (lane 3). The lower band seen in lanes 2 and 3 corresponds to *N*-glycosidase F (34.6 kDa). (B) Pure ATP-DPH (0.014 U, 0.71 μg, lane 1), pure ATP-DPH (0.014 U, 0.71 μg) treated with *N*-glycosidase F (0.66 U) for 12 h (lane 2), pure ATP-DPH (0.014 U, 0.71 μg) treated for 36 h with additions of 1.1 U *N*-glycosidase F at 0, 12 and 24 h (lane 3), buffer of *N*-glycosidase F (lane 4) and potato apyrase (0.16 μg, 0.1 U, lane 5) were subjected to PAGE under native conditions and activity stained using ADP as substrate as described in Section 2.

this enzyme in the preparation is consistent with the identification of the fraction as plasma membrane, since alkaline phosphatase is known to be localized in the cellular membrane anchored via glycosylphosphatidylinositol [29,30].

3.2. *Effect of glycosylation on ATP-DPH activity*

We have previously shown that human placental ATP-DPH is glycosylated [1]. In the present study we tested the type of glycosylation and its effect on the molecular weight and the activity of ATP-DPH. First, we established the optimal conditions for deglycosylation of the native enzyme. Treatment of native ATP-DPH (Fig. 3A, lane 1) with *N*-glycosidase F for 12 h, resulted in a shift of the molecular weight from 82 000 to 80 000 and the appearance of a minor band of 57 500 which suggests that only a very small portion of carbohydrate was removed (Fig. 3A, lane 2). Extension of the incubation time to 36 h with two more additions of *N*-glycosidase F eliminated completely the 82 kDa glycosylated form, and resulted in the formation of the 57.5 kDa deglycosylated product with the concurrent appearance of four other intermediate forms of ATP-DPH (Fig. 3A, lane 3). This result suggests the existence of at least five glycosylated Asn residues in the polypeptide chain of ATP-DPH. Complete deglycosylation of ATP-DPH was achieved using denatured ATP-DPH (boiled in the presence of 0.1% SDS) as substrate of

Table 2
Effect of *N*-glycosidase F treatment on the activity of ATP-DPH

Conditions			ATP-DPH activity, %	
			N-glycosidase F-treated	Control
0 h			100	100
36 h at 37°C			–	76
36 h at 37°C and then dialysis at 0°C	Experiment	1	57	45
		2	75	61
		3	66	53
	Average		66	53

Pure ATP-DPH (0.014 U, 0.71 μg) was incubated for 36 h at 37°C in the absence (control) or in the presence of *N*-glycosidase F with additions of 1.1 units of *N*-glycosidase F at 0, 12 and 24 h of incubation.

N-glycosidase F. This treatment resulted in the formation of a unique product of 57.5 kDa, which is identical with the lower molecular weight form obtained in the partially deglycosylated sample (data not shown). Judging by the decrease of the molecular weight of ATP-DPH (from 82 to 57.5 kDa) after treatment with *N*-glycosidase F, it is concluded that placental ATP-DPH is approx. 30% glycosylated.

The activity of the partially deglycosylated sample was tested as described in Section 2. Both glycosidase-treated and non-treated samples were dialysed to eliminate azide and phosphates which interfere with the assay.

After dialysis the *N*-glycosidase F-treated enzyme and the control sample retained 66% and 53% of their activity respectively. An independent control of ATP-DPH incubated at 37°C for 36 h (no dialysis) retained 76% of its activity. These data, summarized in Table 2, suggest that the deglycosylated enzyme retains its activity.

The effect of deglycosylation on the activity of ATP-DPH was also tested using native gel electrophoresis and activity staining. Native ATP-DPH (Fig. 3B, lane 1), ATP-DPH treated with *N*-glycosidase F for 12 h or 36 h (with two more additions of glycosidase F) stained as a single band (Fig. 3B, lanes 2 and 3 respectively) which migrates with the same mobility as the untreated enzyme. The migration profile of these bands seems puzzling since the above samples consist of different molecular weight forms of ATP-DPH (see Fig. 3A). A possible explanation for this result could be that *N*-glycosidase F removes carbohydrates with negative charge and creates a less negatively charged product and a lower molecular weight protein. Such a modification has opposite effects on the parameters which influence the mobility of a protein on a native gel (charge and size), and may explain the observation that the partially deglycosylated enzyme has the same migration pattern as the native one. On the other hand, since we deal with a membrane protein, another interpretation for the migration pattern could be that the purified enzyme, as well as the partially deglycosylated sample, form aggregates that are not resolved by native gel electrophoresis under mild detergent conditions.

However, despite the peculiar migration behavior, all samples show the same intensity, consistent with the results shown in Table 2, suggesting that partial deglycosylation has no significant effect on the activity of ATP-DPH. The lower band seen in Fig. 3B (lanes 2 and 3) is due to the phosphate ions present in the *N*-glycosidase F preparation (Fig. 3B, lane 4). Potato apyrase, which also hydrolyses ADP [31], was used as a control for the resolution efficiency of the native gel (Fig. 3B, lane 5).

4. Discussion

In the present communication two lines of evidence show that human placental ATP-DPH is a plasma membrane enzyme. First, identical distribution pattern of ATP-DPH and 5′-Nase activity is seen in the fractions obtained after differential centrifugation of human placenta. Second, purified plasma membrane fraction was found to contain ATP-DPH activity. The distribution of both enzymes in the subcellular fractions was not as expected compared to the liver plasma membrane markers [19]. This could be due to the homogenization procedure which yields various sizes of plasma membrane fragments precipitable at $10\,000 \times g$ and $100\,000 \times g$ [19], as well as to the peculiarity of the placental tissue.

ATP diphosphohydrolase from other sources has been mainly localized on the plasma membrane [4–7] with the exception of the pancreatic enzyme which was also detected in Golgi transsacules and on zymogen granules of the acinar cells of pig pancreas [8,9]. The location of ATP-DPH on plasma membranes is consistent with the proposed role of this enzyme in the prevention of blood clotting [32,33]. Besides, the placental enzyme [1] exhibits striking similarities with vascular ATP-DPH from umbilical vessels [34]. Vascular ATP-DPH from bovine aorta has been characterized as an ecto-enzyme [6]. The common properties concern the molecular weight, glycosylation, substrate specificity, sensitivity to various inhibitors and detergents, and dependence on divalent cations. In addition, we have observed that both activities migrate with the same mobility in native PAGE (data not shown). Based on the similarity between these enzymes and the location of the placental ATP-DPH on plasma membranes, we postulate that the placental activity may also be an ecto-enzyme.

ATP-DPH from human placenta (82 kDa) has been previously characterized as a glycoprotein [1]. In this study, extensive incubation of this enzyme with *N*-glycosidase F resulted in the elimination of the fully glycosylated 82 kDa enzyme, and formation of a 57.5 kDa limiting product and four other intermediate forms. The partially deglycosylated sample of ATP-DPH was found to retain its enzymatic activity. Complete deglycosylation of native ATP-DPH proved to be a difficult task, also re-

ported for other glycoproteins [35,36]. In contrast, denatured ATP-DPH was efficiently deglycosylated to yield a single band of 57.5 kDa identical to the limiting product of the partially deglycosylated sample. The shift of the molecular weight upon treatment with *N*-glycosidase F shows that the molecule of ATP-DPH is approx. 30% glycosylated. A similar degree of glycosylation was reported for the ATP-DPH from bovine spleen [3]. A number of reports deal with the role of the glycosylation on the catalytic activity of enzymes. However, no general rule seems to exist so far. Some enzymes require the carbohydrates for their activity while in others the sugar part was not found necessary for their function [15]. Therefore, the functional significance of the existence of the sugars in placental ATP-DPH remains to be established.

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