Glycosylation of bovine pulmonary angiotensin-converting enzyme modulates its catalytic properties

Tatiana Orth[a,b], Sergei Voronov[a], Petr Binevski[a], Wolfram Saenger[b], Olga Kost[a,*]

[a] *Chemistry Department, M.V. Lomonosov Moscow State University, Moscow 119899, Russia*
[b] *Institut für Kristallographie, Freie Universität Berlin, Takustr. 6, D-14195 Berlin, Germany*

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Abstract To study the role of the oligosaccharide moiety in the catalytic properties of angiotensin-converting enzyme (ACE), we obtained asialo- and partially deglycosylated ACE by enzymatic treatment of two-domain somatic enzyme from bovine lung. Treated enzymes demonstrated appreciable, but different changes of catalytic properties in the reaction of the hydrolysis of *N*-substituted tripeptides, C-terminal analogs of angiotensin I and bradykinin among them, compared to those for native enzyme. Deglycosylation also altered the catalytic properties of a single N domain of bovine ACE. So, various patterns of glycosylation modulate substrate specificity of somatic ACE and may be the reason for functional heterogeneity of the enzyme.

Key words: Angiotensin-converting enzyme; Deglycosylation; Asialoenzyme; Substrate specificity; N domain; Bovine lung

1. Introduction

Angiotensin-converting enzyme (ACE, peptidyl dipeptidase A, EC 3.4.15.1) plays an important role in blood pressure homeostasis by generating the vasopressor angiotensin II from angiotensin I and by inactivating the vasodepressor bradykinin. The definite function of ACE may depend on its location [1,2]. There are several molecular forms of ACE in the organism, all forms being heavily glycosylated. The degree of glycosylation, sites of glycosylation, and structures of attached oligosaccharide chains depend on the source of ACE [3,4]. Somatic ACE (150–180 kDa) is mainly located in endothelial, epithelial and neuronal cells. This enzyme is composed of two homologous domains (N and C domains) within a single polypeptide sequence, each domain containing its own catalytic site [2,5]. Somatic ACE has been shown to be only *N*-glycosylated [3,6], human and bovine ACE possessing 17 and 16 potential sites for *N*-glycosylation, respectively [7,8]. The testicular isoenzyme (90–110 kDa) associated with mature germ cells [9] is identical to the C domain of somatic ACE except for a small N-terminal region and contains a single active site. The primary structure of testicular human ACE contains eight potential sites for *N*-glycosylation and an extensively *O*-glycosylated sequence in the N-terminal region [4,10]. ACE-like enzymes have also been identified in non-mammalian species, for example, in *Drosophila melanogaster* [11]. Insect ACE comprises a single domain homologous to both N and C domains of somatic ACE, this enzyme has only three *N*-glycosylation sites.

The investigation of a role of glycosylation in the biosynthesis and activity of rabbit and human testicular ACE demonstrated that newly synthesized ACE acquires glycosylation before its cleavage-secretion as complete blockage of glycosylation results in rapid intracellular turnover of underglycosylated ACE. However, testicular ACE synthesized without *O*-linked sugars or without *O*-linked and a part of *N*-linked sugars can undergo normal transport and secretion, this underglycosylated protein was enzymatically active [4,10]. It was shown that insect ACE-like enzyme does not require glycosylation for intracellular transport and secretion, the underglycosylated mutant was less stable but its catalytic properties were unaltered [11]. In somatic ACE, *N*-glycans are suggested to be important in intracellular targeting, in particular for the expression of the membrane-bound form and secretion of the soluble form, and for the protection of the soluble form against hepatic clearance [6]. The influence of glycosylation on catalytic properties of somatic ACE is still unclear. Deglycosylated ACE from pig lung had the same K_{cat} as native ACE for the substrate Hip-His-Leu, but neuraminidase-treated ACE possessed slightly decreased k_{cat} for the same substrate [6]. On the other hand, neuraminidase had no effect on the catalytic properties of ACE from dog lung [12], and slightly increased the enzymatic activity of ACE from hog kidney [13]. The influence of ACE glycosylation on enzyme substrate specificity has not been studied.

We present here data demonstrating that the extent of ACE glycosylation and sialylation of the ACE molecule are important for the enzyme's catalytic properties.

2. Materials and methods

2.1. Reagents

PNGase F (EC 3.5.1.52) from *Flavobacterium meningosepticum* was from Sigma (St. Louis, MO, USA) and Oxford Glycosystems (Abingdon, UK). Neuraminidase (EC 3.2.1.18) from *Vibrio cholerae* was purchased from Serva (Heidelberg, Germany). The substrates FA-Phe-Gly-Gly, Hip-His-Leu and Cbz-Phe-His-Leu were obtained from Sigma (St. Louis, MO, USA), FA-Phe-Ala-Arg was kindly given by Dr. M. Ovchinnikov from the Cardiology Research Centre, Moscow, FA-Phe-Phe-Arg was a kind gift by Prof. V. Pozdnev from the Institute of Biomedical Chemistry, Moscow. ACE from bovine lung was purified to electrophoretic homogeneity by lisinopril affinity chromatography [14], the elution of the enzyme was performed by the change of eluent from 50 mM phosphate buffer, 0.15 M NaCl, pH 7.5, to 50 mM borate buffer, pH 9.5 [15]. The N domain of bovine ACE was obtained by limited proteolysis of somatic enzyme by trypsin (P. Binevski and O. Kost, in preparation).

*Corresponding author. Fax: (7) (095) 9395417.
E-mail: kost@enzyme.chem.msu.su

Abbreviations: ACE, angiotensin-converting enzyme; PNGase F, *N*-glycosidase F; FA-Phe-Gly-Gly, furanacryloyl-L-phenylalanylglycylglycine; FA-Phe-Ala-Arg, furanacryloyl-L-phenylalanyl-L-alanyl-L-arginine; FA-Phe-Phe-Arg, furanacryloyl-L-phenylalanyl-L-phenylalanyl-L-arginine; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; Cbz-Phe-His-Leu, carbobenzoxy-L-phenylalanyl-L-histidyl-L-leucine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2.2. Treatment of ACE with PNGase F

Deglycosylation of native somatic ACE and the ACE N domain by PNGase F was carried out as recommended by Oxford Glycosystems. PNGase F (20–40 units per 0.3 mg of ACE) was used in 50 mM phosphate buffer, pH 7.1, at 25°C for different periods of time (up to 48 h). Deglycosylated ACE was separated from reaction products (oligosaccharide moiety) by Amicon (USA) YM-30 membrane or by gel chromatography on Sephadex G-50, then the enzyme was stored in 20% (w/v%) glycerol at 4°C. We found that neither reaction products nor glycerol affected the activity of deglycosylated ACE. For complete deglycosylation, somatic ACE was preliminarily denatured at 100°C in acetate buffer, pH 4.0, containing 0.3% SDS, then dialysed against 50 mM phosphate buffer, pH 7.1, overnight and deglycosylated as described above. The apparent molecular mass of native and deglycosylated ACE was analyzed on a 7.5% SDS-PAGE. Protein bands were detected by Coomassie blue R-250 staining. The content of carbohydrates in ACE preparations was determined after exhaustive dialysis by the Dubois colorimetric method with glucose as a standard [16] and by monosaccharide analysis. Monosaccharide content was determined after acid hydrolysis and aminomethyl-coumarin labelling as described in [17].

2.3. Treatment of somatic ACE with neuraminidase

0.05 mg of ACE in 50 mM MES, 0.1 mM $CaCl_2$, 0.15 mM NaCl, pH 6.0, was incubated with 0.3 unit of neuraminidase overnight. For further studies, ACE was separated from reaction products by Amicon (USA) YM-30 membrane. The absence of sialic acids in treated ACE was checked by HPLC of *o*-phenylenediamine derivative [18]. Chromatofocusing of ACE preparations was performed on a Mono-P HR column, LKB system (Pharmacia, Sweden).

2.4. Enzyme activity measurements

The rates of catalytic hydrolysis of FA-Phe-Gly-Gly, FA-Phe-Phe-Arg and FA-Phe-Ala-Arg were monitored spectrophotometrically as described in [19]. The rates of catalytic hydrolysis of Hip-His-Leu and Cbz-Phe-His-Leu were determined fluorometrically by *o*-phthalaldehyde modification of His-Leu as a product of the reaction [20]. Kinetic parameters of the hydrolysis were determined based on the concentration of enzyme active molecules in ACE preparations. Titration of ACE active molecules was performed with the specific inhibitor lisinopril as in [21].

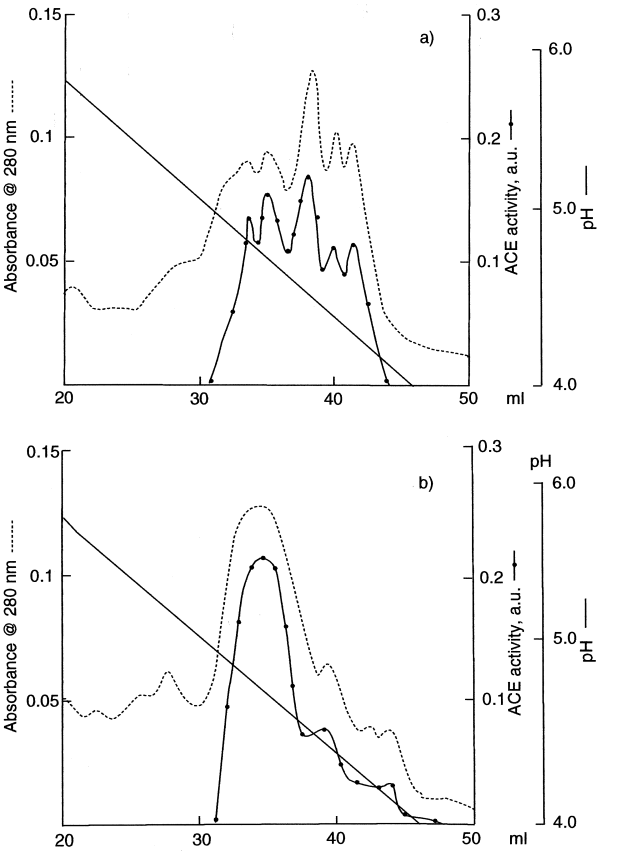

Fig. 2. Chromatofocusing of native (a) and asialo-ACE (b) on a Mono-P column in the pH range from 7 to 4. The activities of ACE were assayed by FA-Phe-Gly-Gly (70 μM) in 50 mM HEPES, 0.3 M NaCl, 1 μM $ZnCl_2$, pH 7.5, at 25°C.

3. Results and discussion

3.1. Enzymatic deglycosylation

Purified bovine pulmonary ACE preparation contained 95% of active molecules as to titration by lisinopril. Carbohydrate content in bovine pulmonary ACE was estimated to be 28% (by weight) by the Dubois method [16] and 32% by monosaccharide analysis [17]. In order to obtain active deglycosylated enzyme, we tried to remove carbohydrates from bovine ACE under non-denaturing conditions by PNGase F, which hydrolyzes all classes of Asn-linked glycans [22]. The apparent molecular mass of PNGase F-treated ACE decreased from 180 kDa to only about 160 kDa (Fig. 1, lanes 1–4), which could be considered a result of incomplete deglycosylation. This was proved by the fact that treatment of denatured enzyme with PNGase F resulted in a decrease of ACE apparent molecular mass from 180 kDa to about 135 kDa (data not shown), which is consistent with the molecular mass of bovine ACE determined from its amino acid sequence [8]. The carbohydrate content in partially deglycosylated ACE was determined to be 12% (by weight). Thus, PNGase F is able to hydrolyze approximately half of all ACE carbohydrate chains in native protein. The increase of PNGase F concentration in the reaction medium, or the prolongation of the time of the reaction, or the use of a mixture of endoglycosidase H and PNGase F (the first enzyme specifically cleaves the linkage between mannose and *N*-acetylglucosamine residues in trimannose cores of *N*-linked sugar chains) failed to raise the degree of deglycosylation. Thus, partially deglycosylated ACE appeared to be not susceptible to further digestion by glycosidases and the main reason for this incomplete deglycosylation seems to be that some cleavage centers in native enzyme are inaccessible to PNGase F due to rigid ACE conformation.

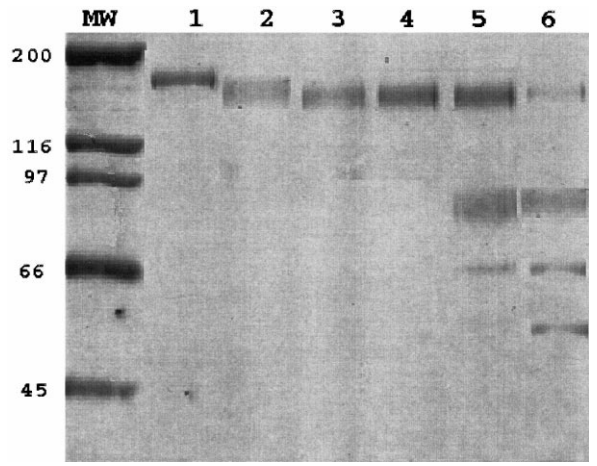

Fig. 1. SDS-PAGE of bovine lung ACE before (lane 1) and after digestion with PNGase F for different periods of time (lane 2, 3 h; lane 3, 5 h; lane 4, 10 h; lane 5, 24 h; lane 6, 36 h).

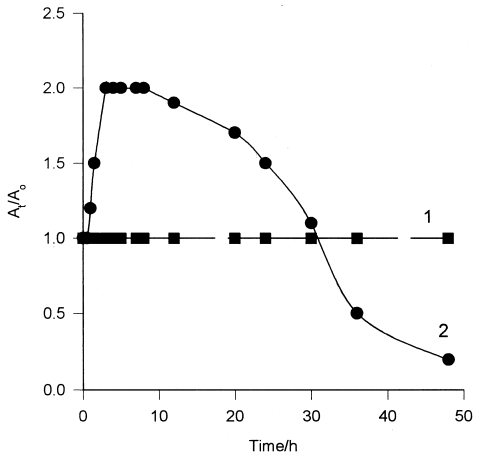

Fig. 3. Time course studies for intact ACE (1) and for ACE upon treatment by PNGase F (2). The ACE activities were assayed as described in the legend to Fig. 2.

Neuraminidase treatment of bovine ACE under non-denaturing conditions resulted in complete loss of sialic acids by protein molecule. As judged by chromatofocusing, bovine ACE appeared to be a mix of active ACE isoforms with a p*I* of 4.3–4.85 (Fig. 2a). Such a microheterogeneity is common for glycoproteins and is caused by a different content of sialic acids in *N*-glycan chains. Desialylation by neuraminidase led to a shift of ACE p*I* with one major enzymatically active peak with p*I* 4.85 (Fig. 2b).

3.2. Effect of deglycosylation on ACE activity and stability

During PNGase treatment, we observed that ACE activity in terms of the hydrolysis of FA-Phe-Gly-Gly markedly increased (Fig. 3) with the extent of deglycosylation followed by the enzyme mobility in SDS-PAGE (Fig. 1, lanes 2–4). It had been shown [23] that oligosaccharides from mucins and even monosaccharides could influence ACE activity. So, there was a possibility that ACE could be activated due to the appearance of free oligosaccharides in the reaction medium. The removal of cleaved sugar chains from treated ACE by exhaustive dialysis, however, did not change enzymatic activity, so it should be attributed to partially deglycosylated ACE. The prolongation of deglycosylation resulted in a fast fall of FA-Phe-Gly-Gly-hydrolyzing activity with a half-life of about 20 h at 25°C (Fig. 3, curve 2). At the same time, the intact enzyme did not inactivate after 40 h preincubation at the same conditions (Fig. 3, curve 1). The loss of catalytic activity of partially deglycosylated ACE was due to degradation of the enzyme as seen by SDS-PAGE (Fig. 1, lanes 5, 6). The reason for this rapid degradation of initially homogeneous purified ACE may be that some kind of contaminating proteinase acts on partially deglycosylated, proteolysis-susceptible ACE. Neuraminidase digestion did not alter ACE stability.

3.3. Effect of deglycosylation on the catalytic properties

We have determined the kinetic parameters of the hydrolysis of several synthetic substrates by native and partially deglycosylated ACE (Table 1). Two of them, Hip-His-Leu and Cbz-Phe-His-Leu, are C-terminal analogs of the ACE native substrate angiotensin I. The substrate FA-Phe-Phe-Arg is a C-terminal analog of another ACE native substrate, bradykinin. As deglycosylated ACE possessed reduced stability, we undertook repeated titration of active molecules in all enzyme preparations during experiments to control possible inactivation. This precaution gave us a possibility to estimate not only V_{max} but real k_{cat} values of the hydrolysis.

We found that the increase of ACE activity towards FA-Phe-Gly-Gly in the course of deglycosylation is due to the increase of k_{cat} value, as K_m remained unchanged. The hydrolysis of Hip-His-Leu and Cbz-Phe-His-Leu by deglycosylated ACE was characterized by decreased values of both k_{cat} and K_m. The most dramatic changes upon deglycosylation were observed in the catalytic parameters of the hydrolysis of FA-Phe-Ala-Arg and FA-Phe-Phe-Arg bearing a positively charged group on C-terminus (Table 1). In these cases, we observed (Table 1) an approximately twofold increase of k_{cat} values but also a 10-fold increase of K_m values, reducing the efficiency of enzyme catalysis (k_{cat}/K_m). Probably, the carbohydrate moiety in bovine lung ACE is involved in the maintenance of the active center conformation making it possible to realize additional contacts of substrate C-terminal Arg with some groups on the native enzyme.

As we noted in Section 1, desialylation could have an effect on ACE activity. To find out whether the change in ACE catalytic properties upon partial deglycosylation was due to the loss of a part of the sialic acids within cleaved oligosaccharide chains, leading to a significant alteration of the charge on the enzyme molecule (Fig. 2), we also studied the influence of neuraminidase treatment on ACE catalysis. We found that desialylation influences ACE catalytic properties, especially k_{cat} values (Table 1). The k_{cat} values of the hydrolysis of FA-Phe-Gly-Gly, Hip-His-Leu, and Cbz-Phe-His-Leu were higher for asialo-ACE, but, unexpectedly, desialylation had no effect on the kinetics of the hydrolysis of the substrate with an additional positive charge, FA-Phe-Phe-Arg.

Table 1
Kinetic parameters of the hydrolysis of synthetic substrates by native, asialo- and partially deglycosylated angiotensin-converting enzyme

Enzyme		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
			FA-Phe-Gly-Gly	
ACE	native	280	1.5	187
	asialo-	650	1.6	406
	deglyc.	517	1.4	369
			Hip-His-Leu	
ACE	native	12	0.56	21
	asialo-	30	0.13	230
	deglyc.	6	0.13	46
			Cbz-Phe-His-Leu	
ACE	native	58	0.27	215
	asialo-	95	0.12	792
	deglyc.	16	0.18	89
			FA-Phe-Ala-Arg	
ACE	native	45	0.027	1667
	asialo-	–	–	–
	deglyc.	100	0.33	303
			FA-Phe-Phe-Arg	
ACE	native	60	0.05	1200
	asialo-	48	0.03	1600
	deglyc.	100	0.6	166

Conditions: 50 mM HEPES, 0.15 M NaCl, 1 μM $ZnCl_2$, pH 7.5, 25°C.

Table 2
Kinetic parameters of the hydrolysis of synthetic substrates by native and partially deglycosylated N domain of angiotensin-converting enzyme

Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
		FA-Phe-Gly-Gly	
N domain native	279	1.4	199
N domain deglyc.	341	1.25	273
		Hip-His-Leu	
N domain native	12	0.5	24
N domain deglyc.	27	0.3	90
		Cbz-Phe-His-Leu	
N domain native	120	0.15	800
N domain deglyc.	300	1.1	272
		FA-Phe-Phe-Arg	
N domain native	45	0.04	1125
N domain deglyc.	50.5	0.12	420

Conditions: 50 mM HEPES, 0.15 M NaCl, 1 μM $ZnCl_2$, pH 7.5, 25°C.

Thus, both partial deglycosylation and desialylation differently affect ACE substrate specificity, and the changes of catalytic properties caused by deglycosylation do not connect with that caused by desialylation. It is likely that the oligosaccharide moiety influences ACE catalysis via definite changes in enzyme conformation which, in turn, affect enzyme active site conformation. Although the change in ACE substrate specificity in vitro does not seem drastic, it may be really important in vivo as differently glycosylated ACE forms may participate in pathological states of the organism. It is interesting that the hydrolysis of the substrates Hip-His-Leu and Cbz-Phe-His-Leu, considered C-terminal analogs of natural substrate angiotensin I, was affected by both desialylation and deglycosylation of the ACE molecule, whereas the hydrolysis of FA-Phe-Phe-Arg, considered a C-terminal analog of bradykinin, was not susceptible to the change of local charge on the enzyme molecule but was strongly affected by the removal of oligosaccharide chains.

As we have noticed before, somatic ACE consists of two domains, each of them containing a catalytic center. The location of the sites of potential glycosylation differs in the N and C domains within the molecule of somatic bovine ACE [8], so the deglycosylation could preferably influence the catalytic properties of only one of these domains. To prove this, we obtained a catalytically active N domain of bovine somatic ACE with a single active center. Treatment of the N domain with PNGase F under non-denaturing conditions also resulted in a partial deglycosylation of the protein, the content of carbohydrates in treated protein was about half that in the native N domain. As for somatic ACE, deglycosylation of the N domain led to remarkable changes in kinetic parameters of catalytic hydrolysis of tripeptide substrates (Table 2). However, we could not find a general tendency in the changes of substrate specificity of somatic full-sized ACE and the single N domain upon deglycosylation. So, the changes of kinetic parameters of two-domain somatic ACE are not attributed to only one domain.

The data obtained suggest that the oligosaccharide moiety in different forms of bovine ACE contributes to the catalytic processes, and heterogeneity of ACE glycosylation may lead to possible functional heterogeneity of the enzyme.

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