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A Conformational "Fingerprint" of the Angiotensin-Converting Enzyme[1]

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Abstract—The angiotensin-converting enzyme (ACE) is a zinc-dependent metalloproteinase widely occurring in the organism; it metabolizes many peptides and plays a key role in blood pressure regulation and vascular remodeling. This enzyme is expressed as a type-1 membrane glycoprotein on the surface of endothelial and epithelial cells, but is also found in a soluble form in biological fluids. In this study, we used purified ACE from lungs, which is mainly produced by endothelial cells of lung capillaries; ACE from heart, produced by endothelial heart cells and, probably, by myofibroblasts; and ACE from seminal fluid, produced by the epithelial cells of the prostate and epididymis. The pattern of binding of a set of 17 mAbs to different conformational epitopes on the surface of two domains of the human ACE significantly differed for ACEs from different organs. This pattern (the conformational "fingerprint" of ACE) reflects the local conformation of the surface of a particular ACE. The differences in the conformational fingerprints of ACEs expressed by different cell types, or even by similar cells but in different organs, can be explained by the posttranslational modification of ACE protein in these organs and, primarily, different glycosylation of *N*-glycosylation sites Asn25, Asn117, Asn289, Asn666, Asn685, and Asn731. The mass spectrometry of tryptic hydrolyzates of ACEs isolated from different human organs made it possible to reveal, in the composition of different ACEs, *N*-glycosylation sites that are really occupied by glycans, namely, Asn in positions 82, 117, 416, 648, 666, 685, and 731 in ACE from seminal fluid; Asn in positions 117, 648, 666, and 685 in ACE from lungs; and Asn in positions 117, 480, 666, and 685 in ACE from heart. Differences in the plausible structures of glycans in ACE, in particular, at the Asn666 *N*-glycosylation site were demonstrated, which can explain the differences in the efficiency of binding of mAbs to ACE from different organs.

Keywords: angiotensin-converting enzyme, monoclonal antibodies, glycosylation, conformation

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INTRODUCTION

The angiotensin-converting enzyme (ACE, peptidyldipeptidase A; EC 3.4.15.1) is a zinc-dependent peptidase widely occurring in mammalian organisms, which is involved in the metabolism of different biologically active peptides (angiotensin 1, bradykinin, goralatide, and others) and plays a key role in blood pressure regulation and many cardiovascular pathologies. In addition, it is involved in the metabolism of neuropeptides, immune and reproductive functions, intracellular signaling, and other processes [1–3].

ACE is a glycoprotein and an integral type I membrane protein. The enzyme fulfils its main functions in the membrane-bound form on the surface of endothelial, epithelial, and neuroepithelial cells, as well as macrophages and dendritic cells. ACE is converted into a soluble form through the cleavage of the stalk region of the enzyme by the action of an accompanying (though yet unidentified) secretase [4, 5]. Soluble ACE is found in blood, seminal fluid, and other biological body fluids.

The somatic form of the ACE consists of two homologous domains (N and C) within the single polypeptide chain; each of the domains contains an active site [6]. At present, the crystal structures of the individual domains of ACE have been solved [7, 8]; however, the exact three-dimensional structure of the full-size enzyme is unknown. Only models based on the structures of the individual domains [9–11], epitope mapping of ACE [9], and the electron microscopy data [10] have been developed.

Earlier, a set of 16 mAbs recognizing the conformational epitopes on the surface of the human ACE have been obtained [9, 12–17]. The studies showed that the

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Abbreviations: ACE, angiotensin-converting enzyme; mAb, monoclonal antibody.

conformational "fingerprint", which characterizes the efficiency of binding of the set of mAbs to different epitopes on the surface of the N- and C-domains of ACE is a sensitive marker of changes in the local conformation of the enzyme surface, which can alter during denaturation and the binding of inhibitors as well as protein and low-molecular-weight effectors [13, 15, 18, 19]. It was shown that the conformational "fingerprint" of ACE is a stable characteristic of the enzyme in the blood of healthy donors [20, 21], where it enters mainly from lung capillaries [22]. However, during the development of diseases, this "fingerprint" can significantly change, which we observed in sarcoidosis [20] and uremia [21]; this is associated with ACE supply into blood from cells other than pulmonary endothelial cells, e.g., from macrophages of sarcoid granulomas.

Differences in the conformation of the surface of ACE molecules expressed in different cell types can be caused by post-translational modification, primarily the different glycosylation of the enzyme, which, in proteins synthesized in different tissues, can differ by the degree of glycosylation of molecules, the position of polysaccharide chains at different glycosylation sites, and by the structure of glycans. It is known that a molecule of human ACE contains 17 potential glycosylation sites [6], but the information on the real glycosylation of human ACE is very limited. A thorough study of ACE glycosylation was performed only on recombinant single-domain ACE (C-domain) expressed in Chinese hamster ovary cells [23]. It was shown in this study that most glycans are complex-type biantennary chains. On the other hand, it was shown that somatic ACE from the human seminal fluid can contain seven oligosaccharide chains of which five chains are complex-type structures, and two chains are of the high-mannose type [24].

At present, the binding epitopes of 16 mAbs to conformational epitopes on the surface of ACE have been characterized; almost each epitope contains a potential glycosylation site. Thus, from the differences in the binding of a particular mAb to ACEs from different sources, it is possible to determine what regions of the protein surface differ in different ACEs and what glycosylation site may be responsible for these differences.

In the present work, we compared the conformational "fingerprints" of ACEs expressed by different cells in the human organism and tried to correlate the differences in the binding of mAbs to these ACEs with the features of their glycosylation.

RESULTS AND DISCUSSION

We have characterized the efficiency of the binding of a panel of conformational mAbs to different epitopes on the surface of N- and C-domains of the human ACE with purified preparations of ACEs expressed by different cell types of the body. ACE from seminal fluid (where it enters from the epithelial cells of the prostate and epididymis [25–27]), ACE from lungs (the main source is the endothelial cells of pulmonary capillaries [22]), and ACE from heart (endothelial cells and, probably, myofibroblasts of the heart [28, 29]) were used. As seen from the data shown in Fig. 1, the relative efficiency of binding of mAbs to purified ACE samples isolated from different human tissues and biological fluids is very different; i.e., the accessibility of these enzymes to mAbs (surface conformation) is different.

Most probably, the differences in the binding of a particular mAb to ACEs from different tissues are caused by the presence or absence of glycan in the binding epitope of this mAb on the surface of ACE and also by glycan structure. The positions of binding epitopes for mAbs in both ACE domains are known [9, 12–17]; therefore, the following conclusions about possible differences in the degree and the type of glycosylation of ACE from different sources can be drawn:

(1) An analysis of the binding of mAbs to ACEs from lung and seminal fluid suggests that different glycosylation of ACE in endothelial and epithelial cells occurs at the following *N*-glycosylation sites: Asn25 in the epitopes for mAbs BB9, 3A5, and i1A8 [14], as well as Asn289 in overlapping epitopes for mABs i2H5 and 6A12 on the N-domain of ACE [14, 15]; Asn666 in the binding epitope for mAb 1E10, Asn685 in the epitope for mAb 2H9, and Asn731 in the epitopes for mAbs 1B8 and 3F10 on the C-domain of the enzyme [9].

(2) An analysis of the binding of mAbs to ACEs from lung and heart suggests that different glycosylation of these ACEs occurs at the following *N*-glycosylation sites: Asn25 in the binding epitopes for mAbs BB9, 3A5, and i1A8 [14], as well as Asn117 in the binding epitope for mAb 5F1 on the N-domain of ACE [16]; Asn666 in the binding epitopes for mAbs 1E10 and 4E3, as well as Asn731 in the binding epitopes for mAbs 1B8 and 3F10 on the C-domain of the enzyme [9].

In addition, the different efficiency of the binding of mAb 1B3 to different ACEs may also be due to the differences in the glycosylation of residue Asn731, which is localized near the epitope for this mAb [9, 13].

It should be emphasized that differences in the binding of ACE from the seminal fluid and ACE from lungs and, correspondingly, of ACE from heart and ACE from lungs (Fig. 1) do not coincide. Thus, the conformational "fingerprint" of the ACE is an individual characteristic of the ACE produced by a particular cell type or even one type of cell (the lungs and the heart) but in different tissues.

We tried to demonstrate that possible differences in the glycosylation of ACE from seminal fluid, lung, and heart may be a probable molecular basis of differences in the binding of mAbs to these ACEs. For this purpose, three purified ACE samples were subjected to trypsinolysis, and the resulting peptides were analyzed

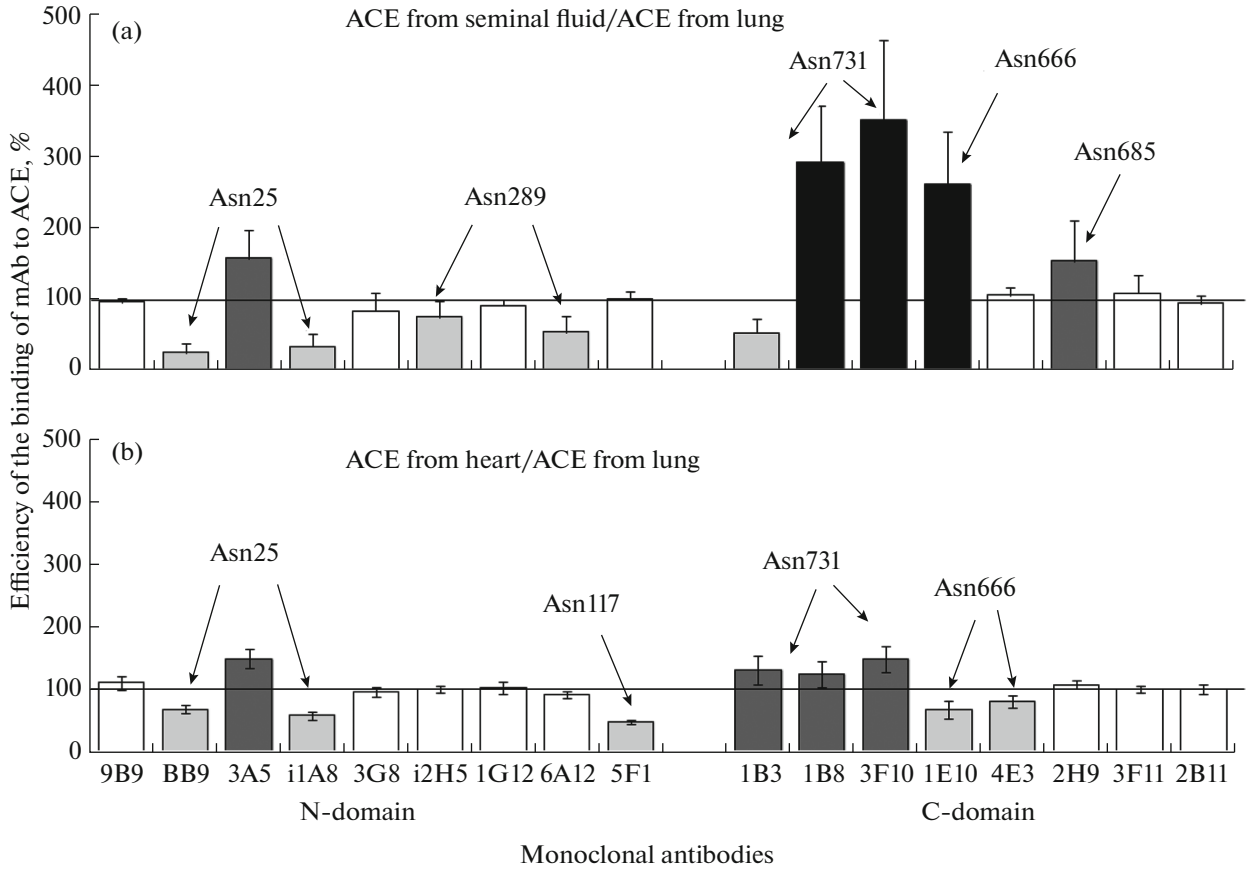

Fig. 1. Immunological characterization of different ACEs. Seventeen mAbs were used for the binding of human ACE. The efficiency of the binding of human ACE with a mAb was estimated by measuring the activity of the enzyme in the plate in complex with the mAb using Z-Phe-His-Leu as a substrate. The data are given as the ratio of the efficiency of the binding of ACE from seminal fluid (a) and heart (b) to the binding of ACE from lung, expressed in percent, for the most clear illustration of the differences in the conformational fingerprints of different ACEs. Colored columns designate the statistically significant data, $p \le 0.05$.

by MALDI-TOF mass spectrometry. ACE samples from seminal fluid and lungs, obtained by two isolation procedures, were analyzed separately, and the results for each enzyme were pooled to have a full picture. Because the content of ACE in heart tissue is low (the activity of the enzyme in heart tissue homogenates was one order of magnitude lower than in lung homogenates prepared in a similar manner), the heart ACE in amounts necessary for the electrophoresis and mass spectrometry was accumulated by several isolation procedures.

After the detection of peptides corresponding to the unglycosylated fragments of the amino acid sequence of the soluble ACE (1203 amino acid residues), it appeared that they account for 62% of the total sequence in the case of ACE from seminal fluid, 69% in the case of the enzyme from lungs, and 58% in the case of the enzyme from heart. The data on unglycosylated peptides identified by the program FindPept in tryptic hydrolyzates of ACE from different human tissues are given in Table 1. In the hydrolyzate of ACE from seminal fluid, unglycosylated peptides containing four potential glycosylation sites: Asn131, Asn494, Asn666, and Asn1196 were found (designated in Table 1 by bold). The hydrolyzate of ACE from lungs contained the same peptides and also a peptide corresponding to sequence 701–750 containing potential glycosylation site Asn731; hydrolyzate of ACE from heart contained, along with four abovementioned peptides, a peptide corresponding to the ACE fragment 74–90, which involved Asn84 (Table 1).

It should be noted that, if the position of a peak in the mass spectrum corresponded to both a glycosylated and an unglycosylated peptide, we assigned this peak only to the unglycosylated peptide. Thereby, we did not take the possible occurrence of partially glycosylated glycosylation sites within ACE into account and tightened the requirements for the detection of possible glycosylated peptides. The only exception were peptides 661–677, which contain the potential *N*-glycosylation site Asn666 in the hydrolyzates of the ACE from the seminal fluid and the lungs (Table 1). The intensity of peaks in the mass spectra that corresponded to this unglycosylated peptide was extremely

Table 1. $[M + H]^+$ ions of unglycosylated peptides in the mass spectra of tryptic hydrolyzates of ACEs from seminal liquid, lung, and heart

ACE from seminal fluid			ACE from lung			ACE from heart		
peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z
53–71[c]	2191.1	2191.1	53–71[c]	2191.1	2191.1	53–71[c]	2191.1	2191.1
54–71	2035.0	2035.0	54–71	2035.0	2035.0			
						74–90[c]	2205.1	2205.1
91–96	628.4	628.4	91–96	628.4	628.4			
97–107	1084.7	1084.6	97–107	1084.6	1084.6	97–107	1084.6	1084.6
127–132[a]	744.4	744.4	**127–132[a]**	744.4	744.4	**127–132[a]**	744.4	744.4
133–151[a]	2135.1	2135.0	133–151[a]	2135.1	2135.0	133–151[a]	2135.0	2135.0
152–187	4117.2	4117.0	152–187	4117.1	4117.0	152–187	4116.9	4117.0
152–187[b]	4132.9	4133.0	152–187[b]	4133.0	4133.0			
188–199	1416.6	1416.6	188–199	1416.6	1416.6	188–199	1416.6	1416.6
200–230	3837.8	3837.9	200–230	3837.9	3837.9	200–230	3837.8	3837.9
231–236[c]	808.5	808.5	231–235[c]	652.4	652.4			
			231–236[c]	808.4	808.5	231–236[c]	808.4	808.5
			232–236[c]	652.4	652.4			
			237–240	510.2	510.2			
237–245[c]	1169.6	1169.6						
241–245	678.4	678.4	241–245	678.4	678.4	241–245	678.4	678.4
296–326	3526.7	3526.7	296–326	3526.7	3526.7	296–326	3526.6	3526.7
296–326[b]	3542.7	3542.6	296–326[b]	3542.7	3542.6	296–326[b]	3542.6	3542.6
327–340[a]	1767.8	1767.8	327–340[a]	1767.8	1767.8	327–340[a]	1767.8	1767.8
327–341[a,c]	1895.9	1895.9	327–341[a,c]	1895.9	1895.9	327–341[a,c]	1895.9	1895.9
341–344[c]	565.3	565.3	341–344[c]	565.3	565.3			
342–346[c]	678.4	678.4	342–346[c]	678.4	678.4	342–346[c]	678.4	678.4
						347–350	507.1	507.2
351–373	2821.3	2821.3	351–373	2821.4	2821.3	351–373	2821.3	2821.3
351–373[b]	2837.3	2837.3	351–373[b]	2837.4	2837.3	351–373[b]	2837.3	2837.3
351–380[c]	3601.9	3601.9						
374–380	799.5	799.5	374–380	799.5	799.5			
374–381[c]	955.5	955.6	374–381[c]	955.5	955.6			
381–407[c]	2852.5	2852.5	381–407[c]	2852.5	2852.5	381–407[c]	2852.5	2852.5
382–407	2696.4	2696.4	382–407	2696.4	2696.4	382–407	2696.3	2696.4
408–413	686.5	686.4	408–413	686.4	686.4	408–413	686.4	686.4

Table 1. (Contd.)

ACE from seminal fluid			ACE from lung			ACE from heart		
peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z
428–458[b]	3640.9	3640.8						
433–446	1724.9	1724.9	433–446	1724.9	1724.9	433–446	1724.9	1724.9
			433–458[c]	3052.6	3052.6	433–458[c]	3052.6	3052.6
447–453	808.4	808.4	447–453	808.4	808.4	447–453	808.4	808.4
			454–458	557.3	557.3			
459–467	1362.7	1362.6	459–467	1362.6	1362.6	459–467	1362.6	1362.6
468–479[c]	1362.7	1362.7	468–479[c]	1362.7	1362.7	468–479[c]	1362.6	1362.7
470–479	1133.6	1133.6	470–479	1133.6	1133.6	470–479	1133.6	1133.6
490–500	1342.8	1342.7	**490–500**	1342.7	1342.7	**490–500**	1342.7	1342.7
490–517[c]	3429.5	3429.8	**490–517**[a,c]	3500.9	3500.8			
501–532[c]	3837.8	3837.8	501–532[c]	3837.9	3837.9	501–532[c]	3837.8	3837.8
518–532[a]	1821.8	1821.8	518–532[a]	1821.8	1821.8	518–532[a]	1821.8	1821.8
						518–541[c]	2663.3	2663.3
542–557[c]	1826.0	1826.0	542–557[c]	1826.0	1826.0	542–557[c]	1826.0	1826.0
542–572[c,b]	3405.9	3405.8	542–572[b,c]	3422.0	3421.9			
543–557	1697.9	1697.9	543–557	1697.9	1697.9	543–557	1697.8	1697.9
			543–572[c]	3278	3277.8			
			543–572[b,c]	3293.9	3293.8			
558–572	1598.9	1598.8	558–572	1598.9	1598.8	558–572	1598.9	1598.8
						558–572[b]	1614.8	1614.8
623–629	957.5	957.4	623–629	957.4	957.4	623–629	957.4	957.4
661–677[c]	1973.8	1974.0	**661–677**[c]	1973.9	1974.0			
671–676	695.4	695.3	671–676	695.4	695.3	671–676	695.4	695.3
694–700[c]	887.5	887.5	694–700[c]	887.5	887.5			
695–700	759.4	759.4	695–700	759.4	759.4			
701–713	1475.8	1475.7	701–713	1475.8	1475.7	701–713	1475.7	1475.7
			701–750[a,c]	5579.1	5578.7			
			750–762[c]	1751.9	1751.9	750–762[c]	1751.9	1751.9
750–762[c,a]	1751.9	1751.9						
750–764[c]	1994.9	1994.9	750–764[c]	1995.0	1995.0			
751–762	1623.8	1623.8	751–762	1623.8	1623.8	751–762	1623.7	1623.8
751–767[c]	2151.1	2151.0	751–767[c]	2151.1	2151.0	751–767[c]	2151.1	2151.0
768–775	979.6	979.6	768–775	979.6	979.6	768–775	979.6	979.6
776–785	1176.7	1176.6	776–785	1176.7	1176.6	776–785	1176.6	1176.6

Table 1. (Contd.)

ACE from seminal fluid			ACE from lung			ACE from heart		
peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z	peptides	found values of m/z	calculated values of m/z
786–797	1352.6	1352.6	786–797	1352.6	1352.6			
798–811	1697.9	1697.8	798–811	1697.9	1697.8	798–811	1697.8	1697.8
798–811[b]	1713.8	1713.8	798–811[b]	1713.9	1713.8			
812–828	2117.2	2117.2	812–828	2117.2	2117.2	812–828	2117.1	2117.2
829–833[c]	652.4	652.4	829–833[c]	652.4	652.4			
834–883	5561.3	5561.7	834–883	5562.0	5561.7			
884–889	744.4	744.4	884–889	744.4	744.4	884–889	744.4	744.4
890–893[c]	581.3	581.3	890–893[c]	581.3	581.3			
915–924	1133.6	1133.6	915–924	1133.6	1133.6	915–924	1133.6	1133.6
940–944[c]	678.4	678.4	940–944[c]	678.4	678.4	940–944[c]	678.4	678.4
945–971[a]	3305.5	3305.6	945–971[a]	3305.6	3305.6	945–971[a]	3305.5	3305.6
972–978	783.5	783.5	972–978	783.5	783.5			
979–1001	2309.2	2309.2	979–1001	2309.3	2309.2	979–1001	2309.2	2309.2
1002–1025	2693.3	2693.3	1002–1025	2693.3	2693.3	1002–1025	2693.3	2693.3
1002–1025[b]	2693.3	2693.3	1002–1025[b]	2709.5	2709.3	1002–1025[b]	2709.3	2709.3
1031–1044	1755.0	1754.9	1031–1044	1754.9	1754.9	1031–1044	1754.9	1754.9
1045–1077[c]	4066.0	4066.1	1045–1077[c]	4066.1	4066.1			
1047–1054	866.5	866.5	1047–1054	866.5	866.5			
1047–1065[c]	2372.1	2372.1	1047–1065[c]	2372.2	2372.1			
1047–1077[a,c]	2372.1	2372.1						
1055–1065	1524.7	1524.7	1055–1065	1524.7	1524.7	1055–1065	1524.7	1524.7
1068–1077	1129.6	1129.6	1068–1077	1129.6	1129.6			
1078–1087	1035.5	1035.5	1078–1087	1035.5	1035.5	1078–1087	1035.5	1035.5
1088–1098	1315.7	1315.7	1088–1098	1315.7	1315.7	1088–1098	1315.7	1315.7
1099–1125	3089.4	3089.5	1099–1125	3089.5	3089.5			
			1133–1137	560.3	560.3			
1181–1189	1056.5	1056.5	1181–1189	1056.5	1056.5			
1190–1202	1533.7	1533.8						
1190–1203	1689.8	1689.8	**1190–1203**	1689.8	1689.8	**1190–1203**	1689.8	1689.8

[a] Cysteine modified by acrylamide.

[b] Oxidized methionine.

[c] Contains one or two bonds that were not cleaved by trypsin.

Peptides containing potential *N*-glycosylation sites are shown in bold.

low, especially in the case of ACE from lungs; therefore, the assignment of this site to unglycosylated was questionable.

An analysis of the mass spectra of peptides using the program GlycoMod revealed several peaks corresponding to possible glycosylated peptides for all three ACEs. Because the search for possible glycopeptides using the program GlycoMod is based on a comparison of the experimentally found molecular masses of peptides with the calculated, several false glycopeptides with rather exotic structures of glycans were obtained. In the subsequent analysis, these glycopeptides were not considered, and only glycans corresponding to the structures deposited in the database UniCarbKB were analyzed.

The results are presented in Tables 2–4. It is seen that, in some cases, the experimentally found masses could be assigned to glycopeptides containing the same potential glycosylation site, which, however, carried glycans of different structure. Thus, for Asn82 in ACE from seminal fluid, two glycan structures are possible (Table 2), and for Asn117 five glycan variants are possible, which differ in the number of antennae, the presence of fucose, and the degree of sialylation or the nature of sialic acid (*N*-acetylneuraminic or *N*-glycolylneuraminic). In some cases, the same mass could be assigned to glycopeptides containing different glycosylation sites. These variants are given at the end of Tables 2 and 3.

Nevertheless, it can be assumed that, in the molecules of ACE from seminal liquid, asparagine residues in positions 82, 117, 416, 648, 666, 685, and 731 are glycosylated (in all, seven glycosylation sites, which is consistent with the data that ACE from seminal liquid contains seven oligosaccharide chains [24]). The glycosylation of Asn480 site is also not ruled out (Table 2). In molecules of ACE from lungs, Asn residues in positions 117, 648, 666, 685, and, probably, Asn480 are glycosylated (Table 3). Finally, in the molecules of ACE from heart, Asn117, Asn480, Asn666, and Asn685 are glycosylated (Table 4). Thus, all three ACEs presumably contain glycosylated Asn residues in positions 117, 666, and 685. It was reported earlier that, in ACE from human kidney, Asn82, Asn117, and Asn480 are glycosylated [23]; and in the molecules of ACE from plasma, Asn residues in positions 480, 666, and 685 are glycosylated [30]. These data correlate with results on ACE from lungs (Table 3), which are known to be the major supplier of ACE into the blood [22, 31].

Unfortunately, in ACE hydrolyzates, no glycopeptides containing the glycosylation site Asn25 were found; this site is incorporated into binding epitopes for mAbs BB9, 3A5, and i1A8 for which significant differences in the binding to the ACE from different sources were demonstrated (Fig. 1). This can be explained by the fact that the corresponding hypothetical tryptic peptide should have a mass (5495 Da) too high to be reliably detected in the mass spectrum. However, peptides containing potential glycosylation sites in positions 666 and 731 were detected, which are parts of the binding epitopes for mAbs whose efficiency of binding to ACE from different tissues significantly differed (Fig. 1). Only in the hydrolyzate of ACE from lungs, an unglycosylated peptide containing Asn731 was found (Table 1), although the intensity of the corresponding peak at 5579 was very low, whereas the possible presence of the corresponding glycosylated peptide was shown for the ACE from seminal fluid (Table 2). Thus, it is probable that the differences in the efficiency of binding of mAbs 1B8 and 3F10 whose epitopes include Asn731 and of mAb 1B3 whose epitope is in close proximity to this residue are due to the presence or absence of glycan at this site.

Because the peptide containing Asn666 was still found among unglycosylated peptides in the hydrolyzates of ACEs from seminal fluid and lungs, though with very low intensity of the corresponding peaks in mass spectra (Table 1), this site in these enzymes should be considered partially glycosylated. In the hydrolyzate of ACE from heart, the unglycosylated peptide containing Asn666 was not detected (Table 1); therefore, it is possible that this site in heart ACE is completely glycosylated.

Possible differences in the glycosylation of the Asn666 site in ACEs from different tissues are presented in Tables 2–4 and illustrated in Fig. 2. Although a great variability of the structures shown in Fig. 2, especially for the ACE from the seminal fluid, it should be noted that, in ACE from seminal fluid and heart, complex- and hybrid-type glycans can be present at site Asn666 (Tables 2, 4 and Fig. 2); though in ACE from lungs, the presence of the high-mannose-type glycan $(Hex)_4 + (Man)_3(GlcNAc)_2$ in the composition of glycopeptide Ile656-Lys670 is possible (Table 3, Fig. 2). Thus, it is the structure of glycan at the *N*-glycosylation site Asn666 that can determine the efficiency of binding of mAbs whose binding epitopes include this site, namely, mAbs 1E10 and 4E3 (Fig. 1).

Thus, the data obtained illustrate the differences in the type of glycosylation of ACE molecules expressed by different cells of the organism, which defines the conformational fingerprint of the surface of ACE and can serve as a molecular basis for the differences in the efficiency of binding of mAbs to the enzyme.

EXPERIMENTAL

The study was carried out in accordance with the Ethical Code of the World Medical Association (Helsinki Declaration) and approved by the Local Commissions of the Bakulev Center of Cardiovascular Surgery, Research Institute of Urology, and University of Illinois at Chicago. The written consent of the donor and his next of kin for the use of tissues or biological fluids in experimental studies has been obtained. The

Table 2. Possible *N*-glycosylated peptides and the corresponding glycan structures in ACE from human seminal fluid

N-Glycosylation sites	Peptides	Found values of *m/z*	Calculated values of *m/z*	Potential structures of glycans
	Glu74-Arg90	3974.0	3973.7	$(Hex)_2(HexNAc)_2(Fuc)_1 + (Man)_3(GlcNAc)_2$ and/or
Asn82				$(Hex)_1(HexNAc)_2(Fuc)_2 + (Man)_3(GlcNAc)_2$
		3933.0	3932.7	$(Hex)_3(HexNAc)_1(Fuc)_1 + (Man)_3(GlcNAc)_2$
	Arg108-Arg120	3494.7	3494.5	$(Hex)_2(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_1(HexNAc)_2(Fuc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$
Asn117		3510.7	3510.5	$(Hex)_2(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_2(HexNAc)_2(NeuGc)_1 + (Man)_3(GlcNAc)_2$
		3203.6	3203.4	$(Hex)_2(HexNAc)_2 + (Man)_3(GlcNAc)_2$
	Gln109-Arg120	5469.1	5469.1	$(Hex)_5(HexNAc)_5(Fuc)_1(NeuAc)_4 + (Man)_3(GlcNAc)_2$
Asn416	Val414-Lys427	3288.5	3288.5	$(Hex)_1(HexNAc)_3 + (Man)_3(GlcNAc)_2$
	Thr630-Lys655	5885.3	5885.4	$(Hex)_5(HexNAc)_4(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or
Asn648				$(Hex)_4(HexNAc)_4(Fuc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$
		5560.3	5560.3	$(Hex)_2(HexNAc)_2(Fuc)_2(NeuAc)_2 + (Man)_3(GlcNAc)_2$
	Asn661-Lys670	3245.4	3245.3	$(Hex)_3(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_2(HexNAc)_2(Fuc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$
		2921.5	2921.2	$(Hex)_1(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$
		3083.6	3083.3	$(Hex)^2(HexNAc)^2\ (NeuAc)^1 + (Man)^3(GlcNAc)^2$ and/or
Asn666				$(Hex)_1(HexNAc)_2(Fuc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$
		3286.6	3286.4	$(Hex)_2(HexNAc)_3(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_1(HexNAc)_3(Fuc)_1\ (NeuAc)_1 + (Man)_3(GlcNAc)_2$
	Asn661-Arg676	3103.2	3103.4	$(Hex)_1(HexNAc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(HexNAc)_1(Fuc)_1 + (Man)_3(GlcNAc)_2$
	Lys677-Lys689	2765.3	2765.3	$(Hex)_2 + (Man)_3(GlcNAc)_2$
Asn685		3259.3	3259.4	$(Hex)_1(HexNAc)_1(Fuc)_1\ (NeuGc)_1 + (Man)_3(GlcNAc)_2$
	Phe678-Arg690	3490.3	3490.5	$(Hex)_2(HexNAc)_2\ (NeuAc)_1 + (Man)_3(GlcNAc)_2$
Asn731	Ile714-Arg749	5885.3	5885.5	$(Hex)_4(HexNAc)_2 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_3(HexNAc)_2(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn416	Ile408-Lys427			$(Hex)_2(HexNAc)_2(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn480 and/or	Asn480-Arg500	4061.1	4060.8	$(HexNAc)_3(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn666	Ile656-Lys670			$(Hex)_4(HexNAc)_3(Fuc)_1 + (Man)_3(GlcNAc)_2$ and/or
				$(Hex)_3(HexNAc)_3\ (Fuc)_2 + (Man)_3(GlcNAc)_2$

Table 3. Possible *N*-glycosylated peptides and glycan structures in ACE from human lung

N-Glycosylation sites	Peptides	Found values of *m/z*	Calculated values of *m/z*	Potential structures of glycans
Asn117	Arg108-Arg120	3349.6	3349.4	$(Hex)_2(HexNAc)_2(Fuc)_1 + (Man)_3(GlcNAc)_2$ and/or $(Hex)_1(HexNAc)_2(Fuc)_2 + (Man)_3(GlcNAc)_2$
	Gln109-Arg120	5469.3	5469.1	$(Hex)_5(HexNAc)_5(Fuc)_1(NeuAc)_4 + (Man)_3(GlcNAc)_2$
Asn648	Thr630-Lys655	5885.4	5885.4	$(Hex)_5(HexNAc)_4(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or $(Hex)_4(HexNAc)_4(Fuc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$
Asn666	Ile656-Lys670	3305.7	3305.5	$(Hex)_4 + (Man)_3(GlcNAc)_2$
Asn685	Lys677-Lys689	2765.4	2765.3	$(Hex)_2 + (Man)_3(GlcNAc)_2$ and/or $(Hex)_1(HexNAc)_5 + (Man)_3(GlcNAc)_2$
	Phe678-Lys689 and/or Phe678-Arg690	3490.5	3490.5	$(Hex)_2(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$
Asn117 and/or **Asn685**	Gln109-Lys126	4238.0	4237.8	$(Hex)_3(HexNAc)_3(Fuc)_1 + (Man)3(GlcNAc)_2$
	Phe678-Arg690			$(Hex)_5(HexNAc)_4(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn480 and/or **Asn666**	Asn480-Lys489	2468.1	2468.0	$(Hex)_3 + (Man)_3(GlcNAc)_2$
	Asn661-Lys670		2468.1	$(HexNAc)_2 + (Man)_3(GlcNAc)_2$
Asn82	Glu74-Arg89	4182.9	4182.8	$(Hex)_3(HexNAc)_3(Fuc)_1(Man)_3(GlcNAc)_2$ and/or $(Hex)_2(HexNAc)_3(Fuc)_2 + (Man)3(GlcNAc)_2$
Asn117 and/or **Asn666**	Gln109-Arg120			$(Hex)_3(HexNAc)_3(Fuc)_1 + (Man)_3(GlcNAc)_2$ and/or $(Hex)_2(HexNAc)_3(Fuc)_2 + (Man)_3(GlcNAc)_2$
	Asn661-Arg676			$(Hex)_3(HexNAc)_4(Fuc)_1 + (Man)_3(GlcNAc)_2$

seminal liquid and the tissues of the heart and lungs served as sources of somatic ACE.

Sixteen mAbs to the human ACE were obtained earlier [9, 12–17], and the BB9 mAb was kindly provided by P.J. Simmons (Brown Foundation of Molecular Medicine, University of Texas Health Science Center, Houston, Texas, United States).

ACE from seminal fluid was isolated by affinity chromatography on lisinopril-Sepharose as described in [32]. ACEs from lung and heart tissues were isolated

Table 4. Possible *N*-glycosylated peptides and glycan structures in ACE from human heart

N-Glycosylation sites	Peptides	Found values of *m/z*	Calculated values of *m/z*	Potential structures of glycans
Asn117	Arg108-Arg120	3349.4	3349.4	$(Hex)_2(HexNAc)_2(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn480	Tyr470-Lys489	4182.8	4182.8	$(HexNAc)_5 + (Man)_3(GlcNAc)_2$
Asn666	Asn661-Lys670	2921.4	2921.2	$(Hex)_1(HexNAc)_2(NeuAc)_1 + (Man)_3(GlcNAc)_2$
	Asn661-Arg676	4182.8	4182.8	$(Hex)_3(HexNAc)_4(Fuc)_1 + (Man)_3(GlcNAc)_2$
Asn685	Phe678-Lys689	4281.9	4281.7	$(Hex)_3(HexNAc)_3(NeuAc)_3 + (Man)_3(GlcNAc)_2$
		3131.4	3131.3	$(Hex)_2(HexNAc)_1(NeuAc)_1 + (Man)_3(GlcNAc)_2$ and/or $(Hex)_1(HexNAc)_1(Fuc)_1(NeuGc)_1 + (Man)_3(GlcNAc)_2$
		2678.3	2678.2	$(Hex)_1(HexNAc)_1 + (Man)_3(GlcNAc)_2$
	Lys677-Lys689	2765.2	2765.3	$(Hex)_2 + (Man)_3(GlcNAc)_2$

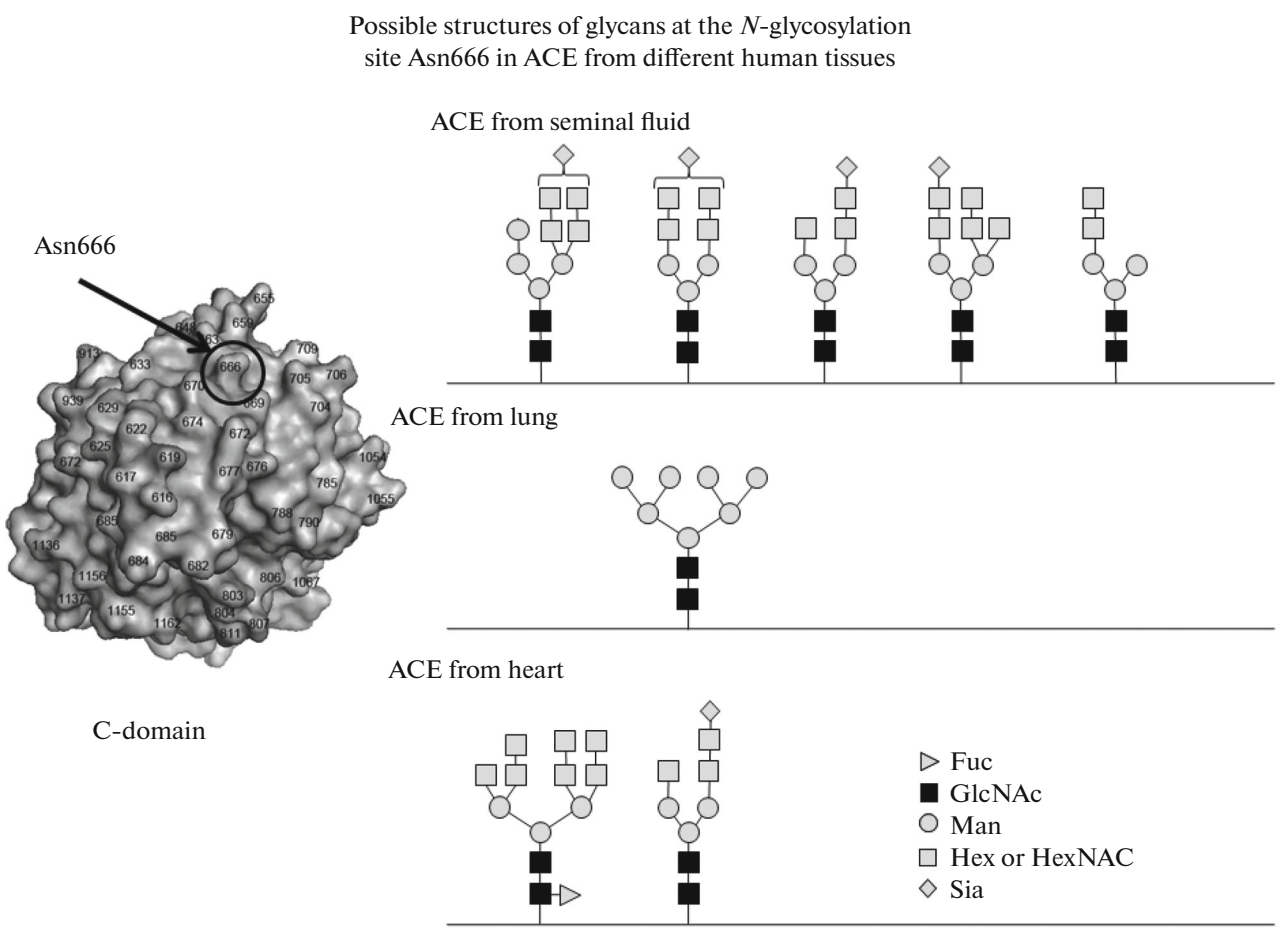

Fig. 2. Variants of glycans in the potential *N*-glycosylation site Asn666 in different ACEs. The mass spectrometry data for the tryptic hydrolyzates of ACEs from human seminal liquid, lungs, and heart were analyzed using the program GlycoMod; in this case, the peaks with masses corresponding to the masses of unglycosylated peptides were not taken into consideration.

and purified by anion-exchange chromatography on DEAE-Toyopearl followed by affinity chromatography as described in [33]. During the isolation of ACE from heart, an additional purification procedure was carried out, which involved several cycles of "concentration–dilution" of ACE solution, obtained by affinity chromatography, on Vivaspin 100 filters (GE Health Care, Sartorius Corp., Bohemia, NY, United States). As a result, ACE from seminal fluid, lungs, and heart homogenous in 7.5% SDS-PAGE were obtained.

The activity of ACE in the seminal fluid, tissue homogenates, and samples of purified enzyme was determined fluorimetrically by a Tecan Infinite M200 microplate fluorescence reader (Tecan, Austria) using two synthetic substrates, 2 mM Z-Phe-His-Leu and 5 mM Bz-Gly-His-Leu (Sigma, United States) at pH 8.3 [34]. The resulting His-Leu product of enzymatic hydrolysis was modified with *o*-phthalaldehyde, and the fluorescence of the adduct was measured at an excitation wavelength of 370 nm and an emission wavelength of 500 nm. Standard fluorescence was determined using His-Leu solutions of known concentration.

The immunological characterization of ACE was performed as follows: 96-well plates Corning (Corning, NY) were covered with antibodies against mouse immunoglobulins (Imtek, Russia), then with the mAb against ACE, and finally with ACE [12]. When comparing the efficiency of binding of mAb to ACEs from different sources, the enzyme solutions were preliminarily equalibrated by enzymatic activity using Z-Phe-His-Leu as a substrate. The plate wells were washed to remove ACE unbound to mAb after which the activity of the enzyme in complex with the mAb was determined by adding the substrate immediately into wells and estimating thereby the amount of bound ACE. Each series of experiments with each ACE sample was carried out at least four times; the binding of the mAb to the enzyme from all tissues was determined in parallel. ACE samples from one tissue type that were obtained by different isolation procedures were analyzed independently, and the results were pooled. The statistical analysis was carried out using the Mann–

Whitney test by the program STATISTICA 6 (StatSoft, Inc., OK).

Trypsinolysis of ACE and mass spectrometry. ACE obtained by two separate isolations of the enzyme from seminal fluid, two isolations of the enzyme from lungs, and several isolations from heart were subjected to electrophoresis under denaturing conditions. All samples obtained were analyzed by mass spectrometry. A piece of an ACE band from electrophoretic gel was transferred to a test tube and the dye was washed out after which the protein was digested by trypsin [23]. Peptides were extracted with 30% acetonitrile containing 0.1% trifluoroacetic acid and subjected to MALDI-TOF mass spectrometry. All mass spectra were recorded on a MALDI-TOF UltrafleXtreme mass spectrometer (Bruker Daltonics, Germany) using 2,5-dihydroxybenzoic acid as a matrix. The data were analyzed by the program MASCOT (www.matrixscience.com) using the SWISS-PROT database and the programs of the site ExPASy, namely, FindPept (http://web.expasy.org/findpept/) and GlycoMod (http://web.expasy.org/glycomod/). The use of the programs MASCOT and FindPept made it possible to determine unglycosylated peptides obtained by the digestion of ACE with trypsin. The peaks in mass spectra that did not correspond to any unglycosylated peptides from the amino acid sequence of ACE were then analyzed using the program GlycoMod to identify possible *N*-glycosylated glycopeptides in ACE. The possibility of the modification of cysteine residues by acrylamide and the oxidation of methionine residues, which are the typical modifications of amino acid residues at protein mass spectrometry, were also included in the data, as well as the possible occurrence of peptides with one or two peptide bonds not cleaved by trypsin.

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