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Artificially glycosylated α-chymotrypsin in reversed micelles of Aerosol OT in octane

A new approach to elucidation of the role of carbohydrate moieties in glycoproteins

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A comparative study of native and artificially glycosylated α-chymotrypsin in reversed micelles of Aerosol OT in octane was carried out. D-Glucosamine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide were used as modifying agents to yield glycosylated enzyme. Unlike the native α-chymotrypsin, the modified protein tended to form reversible oligomeric structures, revealed by the appearance of an additional maximum (characteristic of dimeric forms of protein functioning) as a result of the enzyme catalytic activity being dependent on the AOT hydration degree. Dependence of the enzyme catalytic activity on the surfactant concentration in the case of the modified enzyme was similar to that of glycoproteins, and suggests its membrane affinity. The role of carbohydrate moieties in the functioning of glycoproteins is discussed.

α-Chymotrypsin, artificially glycosylated; Reversed micelle; Aerosol OT; Micellar enzymology; Catalytic activity; Supramolecular structure

1. INTRODUCTION

Carbohydrates are not only widely spread in nature but also show a high variety of forms, which demonstrates their important role in organization and functioning of living matter. However obvious this notion may seem, it was only generally accepted recently (compare, for example [1] and [2]). Apart from carbohydrates themselves, their numerous derivatives, including those with such significant molecular components as proteins (glycoproteins) and lipids (glycolipids and gangliosides), were revealed in nature. The key role of glycanes in cellular and molecular recognition has become common knowledge, and interest in elucidating glycoprotein function grows progressively [3–5]. We may also note here that many biocatalysts are glycoproteins [5,6], and because of a lack of attention to this problem the actual proportion of glycosylated and non-glycosylated enzymes existing in nature is still hard to estimate. The presence of carbohydrate fragments in enzyme molecules is sometimes taken into account when considering their stability, oligomerization, and interaction with biomembranes [5].

There seem to be two main reasons why explaining the role of carbohydrate moieties in enzyme molecules remains a challenge for biochemists. First, experiments of this kind usually involve step-by-step deglycosylation of the enzyme, an extremely complex and costly procedure not necessarily leading to reliable results that can be interpreted unambiguously. Second, and probably more important, modern enzymology, as it is widely known, is a rather artificial field. The properties of enzymes are generally studied in aqueous solutions, that is, in an environment far from native. One cannot therefore exclude the possibility that it is because of the aqueous medium that the effects of the carbohydrate parts of enzymes become negligible.

We propose a novel approach to clarifying the role of glycane in glycoprotein enzymes. First, a simple non-glycosylated and well-characterized enzyme is used as the object, which permits one to vary the carbohydrate components, starting from the simpliest ones, and change their number and chemical nature. Second, the system of reversed micelles [7] is used as the reaction medium for enzymatic reactions since this system has been shown to imitate enzyme–biomembranes interactions. As a first step of this research we present here the results of a comparative study of the catalytic activity of α-chymotrypsin (ChT), native and artificially glycosylated by D-glucosamine, in reversed micelles of Aerosol OT (AOT) in octane.

2. EXPERIMENTAL

2.1. *Materials*

ChT (EC 3.4.21.1) from bovine pancreas (Koch-Light Lab. Ltd.) was used as obtained. The content of the active enzyme in the prepa-

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Abbreviations: AOT, Aerosol OT (sodium bis-(2-ethylhexyl)-sulfosuccinate); ChT, α-chymotrypsin; CI, *N-trans*-cinnamoylimidazole; ATNA, *N*-acetyl-L-tyrosine *p*-nitroanilide; BTNA, *N*-benzoyl-L-tyrosine *p*-nitroanilide; IR, infrared spectroscopy.

ration, estimated by titration with CI (*N-trans*-cinnamoylimidazole) [8], was 56%. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and ATNA (*N*-acetyl-L-tyrosine *p*-nitroanilide) were purchased from Serva. D-Glucosamine was a kind gift from Dr. I. Yamskov (Moscow, Russian Federation). CI and Tris-HCl were Sigma reagents. AOT (Merck) was used without additional purification. According to infrared spectroscopy (IR) data, the preparation contained 0.6 mol of water per mol of the surfactant. This value was taken into account when the total amount of water in the micellar system was calculated ($w_o = [H_2O]/[AOT] \approx 0.6$). All other reagents and solvents were used as obtained from Reakhim.

2.2. *Modification of ChT with D-glucosamine*

ChT (320 mg) was dissolved in 20 ml of 50 mM sodium-phosphate buffer, pH 5.0, containing 150 mM NaCl, and 70 mg of *N*-acetyl-L-tyrosinamide was added to protect the enzyme active site, followed by 247 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and 830 mg of D-glucosamine hydrochloride. The excesses of carbodiimide and D-glucosamine per one exposed carboxylic group of the protein were 10- and 30-fold, respectively. The reaction was carried out for 18 h at 20°C with constant stirring. After the reaction was complete, the modified ChT was separated from low molecular weight compounds using dialysis for 24 h against 1 mM HCl at 4°C. The separation was verified by measuring absorbance at 280 nm and conductivity. The dialyzed product was lyophilized. The modified ChT yield was 236 mg. The glycosylated ChT had 50% of the original activity of the unmodified enzyme, as estimated by the standard titration procedure [8]. Upon polyacrylamide gel electrophoresis under denaturing conditions, the glycosylated ChT preparation gave rise to one narrow band which stained for protein with a molecular weight close to 25 kDa, corresponding to that of native ChT. Isoelectric points of the native and modified proteins, as estimated by standard isoelectric focusing procedure (Bio-Rad, USA), were 8.3 and 9.6, respectively. These results demonstrated that the preparation obtained was homogeneous and devoid of intermolecular linkages. The degree of ChT modification with D-glucosamine, estimated by titration of carboxylic groups with Woodward's reagent [9], was 7 ± 2 mol/mol.

2.3. *Enzyme activity measurements*

All kinetic experiments were performed in a Philips PU-6830 spectrophotometer with the thermostated cell holder at 25°C under steady-state conditions. ChT activity was measured with a non-specific substrate, CI, and specific substrates, ATNA and BTNA (*N*-benzoyl-L-tyrosine *p*-nitroanilide). The values of the catalytic rate constant were evaluated from initial rates of substrate hydrolysis using the Lineweaver–Burk plot.

2.3.1. α-Chymotryptic hydrolysis of CI

In a typical experiment, 40–200 μl of 50 mM Tris-HCl buffer, pH 8.0 (or 1 M of glucose in 50 mM Tris-HCl buffer), and 5 μl of 40 mM solution of CI in acetonitrile were solubilized in 2 ml of 0.2 M AOT solution in octane, and the background and non-enzymatic substrate hydrolysis was recorded. Then 5 μl of ChT stock solution in 1 mM HCl (or glycosylated ChT) were added. After vigorous shaking, the reaction was followed by a decrease in absorbance at 335 nm. Rates of enzymatic hydrolysis of CI were corrected for non-enzymatic hydrolysis. The molar extinction coefficients of CI measured in the micellar systems ranged from 2,000 $M^{-1} \cdot cm^{-1}$ at $w_o = 7$ to 3,000 $M^{-1} \cdot cm^{-1}$ at $w_o = 30$.

2.3.2. α-Chymotryptic hydrolysis of ATNA and BTNA

In a typical experiment, 20–100 μl of 50 mM Tris-HCl buffer, pH 8.5, and 2 μl of 1.4 M ATNA in a DMSO:DMFA (1:1) mixture (or 3 μl of 140 mM BTNA in an dioxane:acetonitrile (1:1) mixture) were solubilized in 2 ml of 0.1 M AOT solution in octane, then 2 μl of ChT (or glycosylated ChT) stock solution of known active site concentration in 1 mM HCl were added. After shaking by hand, the rate of *p*-nitroaniline formation was measured spectrophotometrically at 380 nm. The molar extinction coefficients of *p*-nitroaniline measured in the micellar systems ranged from 8,800 $M^{-1} \cdot cm^{-1}$ at $w_o = 7$ to 9,600 $M^{-1} \cdot cm^{-1}$ at $w_o = 30$.

2.4. *Sedimentation measurements*

The sedimentation coefficients (s) of the reversed micelles containing ChT or glycosylated ChT were measured at 20°C in a Beckman E analytical ultracentrifuge, equipped with a photoelectric scanning device, monochromator, and a multiplexor, using 12 mm bisection cells and an An-G-Ti rotor at 20,000–44,000 rpm. The scanning was carried out at 280 nm. The sedimentation coefficients of buffer-containing or glucose-containing reversed micelles without protein were measured in separate runs. The dependences of s on w_o were analyzed as described [10]. The values of the molecular weight of the protein incorporated into the reversed micelles were calculated from s values as previously described [10].

3. RESULTS AND DISCUSSION

First of all, we compared the catalytic properties of the native and the glycosylated ChT in aqueous medium (Tris-HCl, pH 8.5, using BTNA as the substrate). The values of k_{cat} and K_m for the native and glycosylated ChT were found to be identical, which meant that either the covalent modification of the enzyme surface does not affect its functioning or the effect cannot be detected in water.

However, the situation was different when we used the reversed micelles as a reaction medium. Fig. 1a shows the dependence of the catalytic activity of the native ChT on the hydration degree, w_o, a parameter which determines the size of the inner cavity of a micelle. The results are presented both for the non-specific substrate, CI (the rate-limiting step is deacylation with the rate constant, k_3), and for the specific substrate, ATNA (the rate-limiting step is acylation with the rate constant, k_2). As seen in Fig. 1a, regardless of the nature of the substrate, the ChT activity profiles coincide, and the maximum k_{cat} is observed at $w_o = 10$. According to our concept of geometrical coincidence [11], the solubilized enzyme is most active when the size of the inner water cavity of the reversed micelle is equal to that of the protein molecule, in this case ChT. With the glycosylated ChT, an additional, second optimum appears with the dependence of k_{cat} on w_o, which is typical of oligomeric dissociating enzymes in reversed micelles [13].

The first optimum at $w_o = 12$ observed in Fig. 1b corresponds to the usual monomeric form of the enzyme, the optimum position being slightly shifted compared to the native enzyme (Fig. 1a and b) because of an increase in the size of the protein molecule after glycosylation. The occurrance of the second optimum at $w_o = 20$ may be attributed to an associated enzyme form in this system.

In principle, the size of the aggregate incorporated into reversed micelles can be estimated with a certain precision from the position of the optimum ($r = 34$ Å for $w_o = 20$). However, it is difficult to determine the stoichiometry using only these data since neither the

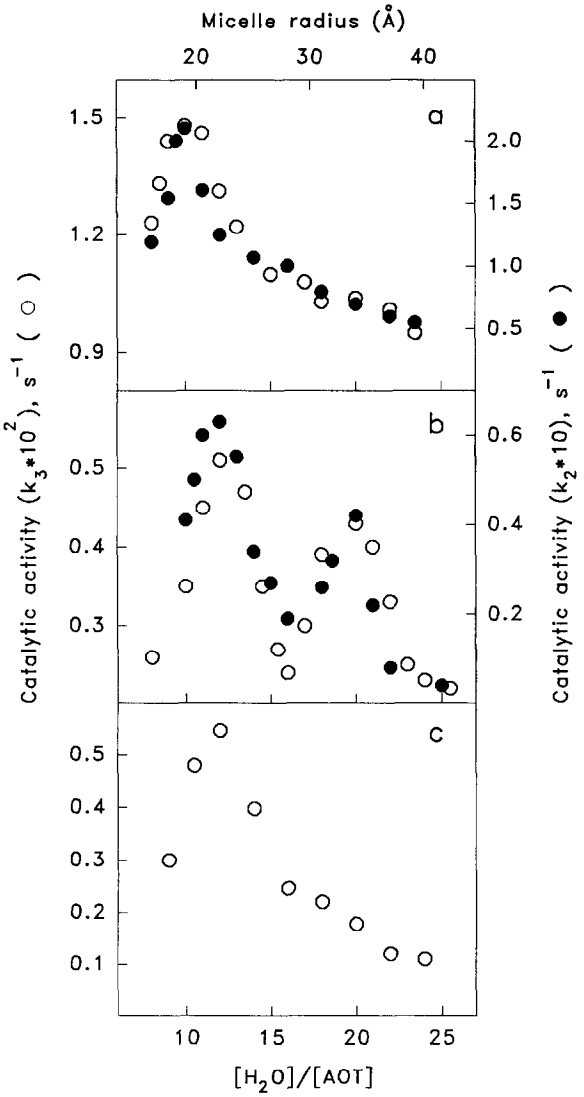

Fig. 1. Profiles of the catalytic activity vs. hydration degree ([H_2O]/[AOT]) for α-chymotrypsin (a) and artificially glycosylated α-chymotrypsin (b, c) in reversed micelles of AOT in octane. In (c) 1 M glucose was added. Substrates: *N-trans*-cinnamoylimidazole, (○); *N*-acetyl-L-tyrosine *p*-nitroanilide, ATNA (●).

packing type nor the way the ChT molecules interact are known. If one assumes that dimerization of a spherical molecule gives rise to an aggregate resembling a sphere or a dumb-bell, the molecule size could be a factor of 1.25 (sphere) or 2 (dumb-bell), with any intermediate case being possible.

To establish the type of the oligomeric ChT form we carried out sedimentation analysis of the protein-containing micellar system. The results indicated that the stoichiometry of ChT incorporation at 2–5 μM enzyme concentration was 1:1 throughout the entire AOT hydration degree range with native ChT and 2:1 at w_o greater than 20 with glycosylated ChT. The latter value suggests dimer formation in the case of glycosylated ChT.

In view of this, we suggest that the glycane moiety of the enzyme contributes to the dimerization. If this is the case, addition of a low molecular weight sugar to the system may weaken the intermolecular interaction within the dimer. Indeed, it can be concluded from a comparison of Fig. 1b and c that the optimum corresponding to the oligomeric form of the enzyme disappears in the presence of 1 M glucose, and the k_{cat} vs. w_o profiles for the modified enzyme resemble those for the native ChT.

We were also able to detect the glucose-induced dissociation of the glycosylated dimeric ChT by means of sedimentation analysis. Thus, the way glucose is brought into the system – covalently or not – can reversibly change the supramolecular organization of the enzyme under study.

On the other hand, the covalently attached carbohydrates may not only strengthen a protein's ability for intermolecular interaction and oligomer formation but may also confer some affinity to membranes, like with natural glycoproteins [11]. It has been shown earlier in this laboratory that the dependence of the catalytic activity on surfactant concentration at w_o = const can be used as a membrane affinity test [11,12]. Fig. 2 shows that native ChT (curve 1) has no membrane affinity and that k_{cat} is independent of surfactant concentration. In the case of the glycosylated derivative, there is, however, a strong dependence of k_{cat} on surfactant concentration (Fig. 2, curve 2). This experimental fact is further evidence of the interaction between glycosylated ChT and the micellar matrix.

Thus, the introduction into a protein molecule of a small number of carbohydrate groups brings about a drastic change in the catalytic properties of the enzyme, due to regulation by the membrane-like matrix. We believe that this approach will be helpful in unravelling the specific roles of glycanes in glycoproteins. To this end, we are planning to vary the nature, number and

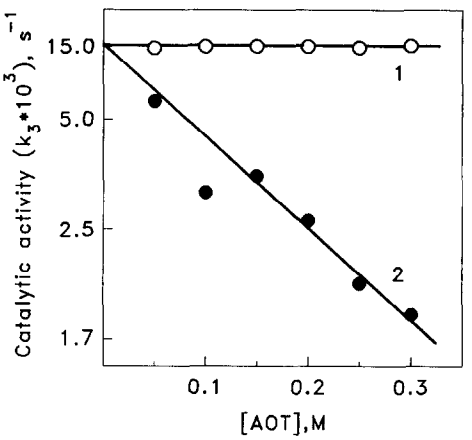

Fig. 2. Dependences of α-chymotrypsin (1) and artificially glycosylated α-chymotrypsin (2) catalytic constants on surfactant concentration in reversed micelles of AOT in octane at w_o = 10 (1) and 16 (2). Substrate: *N-trans*-cinnamoylimidazole, CI.

size of the carbohydrate moieties both on the protein surface and in the micellar matrix.

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