View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Volume 295, number 1,2,3, 73–76 FEBS 10507 December 1991
© 1991 Federation of European Biochemical Societies 00145793/91/$3.50

Subunit separation in reversed micelle system reveals the existence of active centers both on light and heavy γ-glutamyltransferase subunits

Alexander V. Kabanov[1,2], Sergey N. Nametkin[1], Nikolai N. Chernov[3], Natalya L. Klyachko[1] and Andrey V. Levashov[1]

[1]*Laboratory of Micellar Enzymology, Division of Chemical Enzymology, Department of Chemistry, Moscow State University,* [2]*Laboratory of Biopolymer Chemistry, Department of Bioregulation, All-Union Research of Molecular Diagnostics and Therapy* and [3]*Department of Biochemistry, Patrice Lumumba University, Moscow, Russia*

Received 27 September 1991

Regulation of supra-macromolecular composition and catalytic activity of a heterodimeric enzyme, γ-glutamyltransferase, in the system of Aerosol OT (sodium bis(2-ethylhexyl) sulfosuccinate) reversed micelles in octane were studied. Variation of the surfactant hydration degree (parameter, determining dimensions of the polar inner cavity of the micelle) causes a reversible dissociation of the enzyme to light and heavy subunits. Both enzyme subunits possess catalytic activity. The light and heavy subunits of the enzyme were separated on a preparative scale in a reversed micelle system using ultracentrifugation. The active centers of γ-glutamyltransferase were studied using its irreversible inhibitor — AT-125 (L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid). Separation of the γ-glutamyltransferase subunits results in the 'opening' of a new active center located at the heavy subunit. In the dimer form of the enzyme this center is masked and it is not accessible to both substrate and inhibitor molecules.

γ-Glutamyltransferase; Protein subunit; Micellar enzymology; Reversed micelle; Supra-macromolecular structure

1. INTRODUCTION

Application of reversed micelles (RM) systems as reaction medium in enzymology [1] opens new perspectives in the analysis of structure–function relationships in oligomeric enzymes and multi-enzyme supramolecular complexes [2–5]. The size of the inner polar cavity of RM can be widely varied via variation of the surfactant hydration degree, w_0, i.e. [H_2O]/[surfactant] molar ratio [6,7]. This permits to use RM as a matrix microreactor for fine chemical processing of macromolecules entrapped into polar cavities, in particular for regulation of a supramolecular composition of solubilized protein complexes [8]. The possibility of purposefully changing the oligomeric composition of a solubilized enzyme by changing the size of the micellar matrix has recently been demonstrated using a variety of examples: lactic dehydrogenase [2,5], γ-glutamyltransferase [γ-GT] [3], alkaline phosphatase [4], keto-glutarate dehydrogenase [5] and glyceraldehyde-3-phosphate dehydrogenase [5]. The RM matrix permits to produce artificially oligomeric forms of different composition, including those which can not be obtained in homogeneous aqueous solutions, or which can be obtained only under denaturing conditions [2–5]. The basic point is that these forms possess the functional activity in RM systems. Thus, the new strategy for the study of oligomeric enzymes has been formulated [3]. It is based on the study of catalytic and other properties of oligomeric forms produced in RM.

This paper is devoted to the study of a heterodimeric enzyme — γ-GT using the combination of the technique of its subunit separation in RM and inhibitor analysis. The system of AOT RM in octane is used as a medium for γ-GT subunit separation. In order to prove that, contrary to observations made before with the application of traditional methods [9], both light (M_r=21 000) and heavy (M_r=54 000) γ-GT subunits contain similar active centers, the irreversible enzyme inhibitor AT-125 is used.

Abbreviations: γ-GT, γ-glutamyltransferase; AOT, Aerosol OT (sodium bis(2-ethylhexyl)sulfosuccinate); NA, (4-nitro)aniline; CNA, (3-carboxy-4-nitro)aniline; GluNA, γ-glutamyl(4-nitro)anilide; GluCNA, γ-glutamyl(3-carboxy-4-nitro)anilide; AT-125, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; GlyGly, glycylglycine; RM, reversed micelle; PAGE, polyacrylamide gel electrophoresis.

Correspondence address: S.N. Nametkin, Department of Chemical Enzymology, Faculty of Chemistry, Moscow State University, Leninskiye Gory, Moscow 119899, USSR.

2. MATERIALS AND METHODS

2.1. *γ-GT extraction*

The soluble form of γ-GT ((5-glutamyl)-peptide: amino acid 5-glutamyltransferase, EC 2.3.2.2) was extracted from the entwined poorly differentiated hepatoma H-27 using the method [10] which included enzyme solubilization with papain and purification by gel filtration on Sephadex G-150. According to the PAGE data the obtained enzyme preparation was homogeneous. Its catalytic activity

was equal to 30 U/mg (one unit liberates 1 μmol of NA from GluNA per 1 min at 25°C, pH 8.5).

2.2. *γ-GT activity in RM [3]*

In a typical experiment 20 μl of 1 μM γ-GT and 10–120 μl of 0.5–50 mM GluCNA (Sigma) solutions in aqueous buffer were solubilized in 2 ml of 0.1 M AOT (Merck) solution in octane (Reakhim); 25 mM Tris-HCl (pH 8.8) containing 0.1 M GlyGly was used as an aqueous buffer. The formation of free CNA was determined spectrophotometrically (400 nm) at 25°C. The values for CNA molar absorption were determined by an independent experiment. The values $V/[E_0]$ were determined under the conditions of pH-optimum and saturation of the enzyme with substrates (GluCNA and GlyGly).

2.3. *The sedimentation measurements*

Sedimentation coefficients (s) of RM containing the protein were measured as described in [11] at 20°C in an analytical ultracentrifuge 'Beckman E', fitted with a photoelectric scanning device with a monochromator and a multiplexor, using 12 mm bisection cells and a rotor An-G-Ti at 20 000 rpm. The scanning was carried out at 280 nm. The dependencies of s on w_0 were analyzed as described in [5]. The values of the molecular mass (M_r) of the protein incorporated into the RM were calculated from the s values [5].

2.4. *Separation of light and heavy subunits of γ-GT [3]*

Two hundred twenty five microliters of 4 μM γ-GT solution in 25 mM Tris-HCl (pH 7.5) were solubilized in 25 ml of 0.1 M AOT in octane. The micelle fraction containing the heavy subunit was precipitated at 30 000 rpm during 40 min using the MSE Superspeed 65 centrifuge. The supernatant was separated from the sediment. The sediment was solubilized in 10 ml of 0.1 M AOT in octane. The protein contained in the sediment and supernatant was precipitated from the RM system with acetone and analyzed by PAGE.

2.5. *Inhibition of* γ-GT with AT-125

Step I. From 10 to 150 μl of 5.6 mM AT-125 solution were added to 0.3–3 ml of 14 μM γ-GT solution in 25 mM Tris-HCl (pH 7.6). After 2 h incubation at room temperature the enzyme was separated from the excess of inhibitor by gel-filtration on Biogel-P2. The activity of γ-GT in aqueous solution (transpeptidation reaction) was determined by the method analogous to the one described in [12] using L-GluCNA as a substrate.

Step II. Sixty microliters of 100 mM L-Glu(CNA) solution and 72 μl of 50 mM GlyGly solution in 50 mM Tris-HCl (pH 8.8) were added to 60 μl of 23 μM γ-GT solution obtained as described above (Step I). After 2 h incubation at room temperature this mixture was solubilized in 6 ml of 0.1 M AOT. The activity of enzyme in the RM system was determined as described in section 2.2.

Step III. The γ-GT subunits were separated as described in section 2.4. The subunit activity was determined either in the RM system or in aqueous solution. In the latter case the subunits were precipitated from the micellar solution with acetone and then dissolved in aqueous buffer.

Step IV. The heavy subunit was incubated with AT-125 using the procedure analogous to the one described above (Step I). Its activity was determined either in aqueous solution or in RM system.

3. RESULTS AND DISCUSSION

3.1. *γ-GT catalytic activity versus hydration degree dependence*

The profile of γ-GT activity in AOT RM in octane (Fig. 1A, curve 1) reveals several maxima observed at w_0=11, 17 and 26. This picture differs from those observed for monomeric or non-dissociating oligomeric enzymes, characterized by the existence of a single maximum [13,14]. The hypothesis explaining such unusual γ-GT behavior has been proposed recently [3]. It is based on the fact that the reversible dissociation of this heterodimeric enzyme to the catalytically active subunits proceeds in AOT RM. The observed maxima correspond to the functioning of light (w_0=11) and heavy (w_0=17) γ-GT subunits and of their dimer (w_0=25) [3].

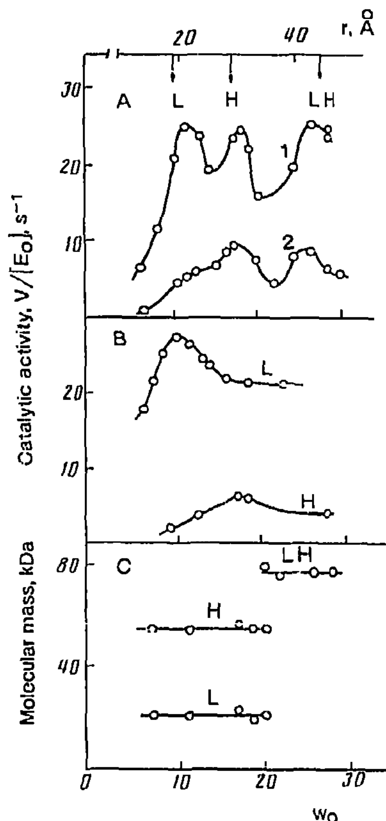

Fig. 1. (A) The dependence of the catalytic activity ($V/[E_0]$) of γ-GT (transpeptidation reaction) on the hydration degree in the system of AOT RM in octane. The data for native γ-GT (1) and γ-GT, treated with AT-125 (2) are presented. The scale of the micelle inner cavity (r) is presented [6]. Values of the radii of the light (L) and heavy (H) subunits as well as their dimer (LH) are shown by arrows. (B) The dependence of the catalytic activity ($V/[E_0]$) of the light (L) and heavy (H) γ-GT subunits (transpeptidation reaction) on the hydration degree. (C) Change of the oligomeric composition of γ-GT in AOT RM in octane. Values of molecular masses of the protein solubilized in RM were calculated from experimental sedimentation coefficients of protein-filled micelles [5].

Sedimentation data (Fig. 1C) reveal that at w_0>20 γ-GT exists in the RM system exclusively in the form of the dimer. At lesser hydration degrees, two different populations of protein-containing micelles are detected on the sedimentogram: micelles with light (L) and heavy (H) subunits. The considerable difference in sedimentation coefficients of these two micelle types permits the separation of light and heavy γ-GT subunits. Micelles containing the heavy subunit were precipitated using preparative centrifugation, while micelles with the light

subunit remain in solution. According to the PAGE data (not shown in figures) the obtained precipitate only contained heavy γ-GT subunit. The light subunit and a small amount of the heavy one (not more than 5% (w/w)) were found in the supernatant.

Catalytic activity dependencies of obtained light and heavy subunits on the hydration degree are presented in Fig. 1B. These profiles represent curves with only one maximum. In the case of the light subunit, the catalytic activity optimum is observed at $w_0=11$, in the case of the heavy one at $w_0=17$. On mixing the micellar solutions containing light and heavy subunits the profile of the catalytic activity becomes analogous to the one presented in Fig. 1A (curve 1); the third maximum corresponding to the γ-GT dimer appears at $w_0=26$ (data not shown in figure). Hence, the reconstruction of the oligomeric form of the enzyme takes place.

3.2. *Inhibition of γ-GT with AT-125*

The γ-GT active center is believed [9] to contain at least three different sites: one so-called donor site, which binds L(D)-glutamyl-containing substrate, and two acceptor sites which bind nucleophiles attacking the substrate molecules. The localization of these sites has not been finally established. It is, however, assumed [17,18] that the donor part is located on the light enzyme subunit, and that both light and heavy subunits participate in the formation of the acceptor sites. The data obtained by us contradict this assumption. The fact that both subunits possess the catalytic activity, in particular, in the transpeptidation reaction (Fig. 1B), suggests that donor sites exist both on the light and heavy subunits. To prove the existence of several catalytic sites in γ-GT, we carried out the inhibitor analysis using its specific irreversible inhibitor AT-125.

The scheme illustrating the results of this experiment is presented in Fig. 2. Incubation of γ-GT with AT-125 in aqueous solution leads to the irreversible loss of enzyme activity (Fig. 2, Step I), which coincides with the previously described data [17,18]. After solubilization of AT-125-treated γ-GT in the RM system under conditions ($w_0=17$) when subunit dissociation takes place (Fig. 1C), the partial restoration of enzyme activity (up to 40% of the initial level) is observed (Fig. 2, step II). At the same time the profile of the γ-GT activity dependence on the hydration degree changes considerably: elimination of the maximum at $w_0=11$, corresponding to the light subunit functioning, takes place (Fig. 1A, curve 2).

Subunits of AT-125-treated γ-GT were separated via ultracentrifugation in the RM system as described above. Catalytic activities of the subunits were studied either in AOT RM in octane or in aqueous solution after subunit precipitation from the RM system with acetone. This permitted to establish that just the heavy subunit possesses catalytic activity whereas the light subunit under the experimental conditions studied is

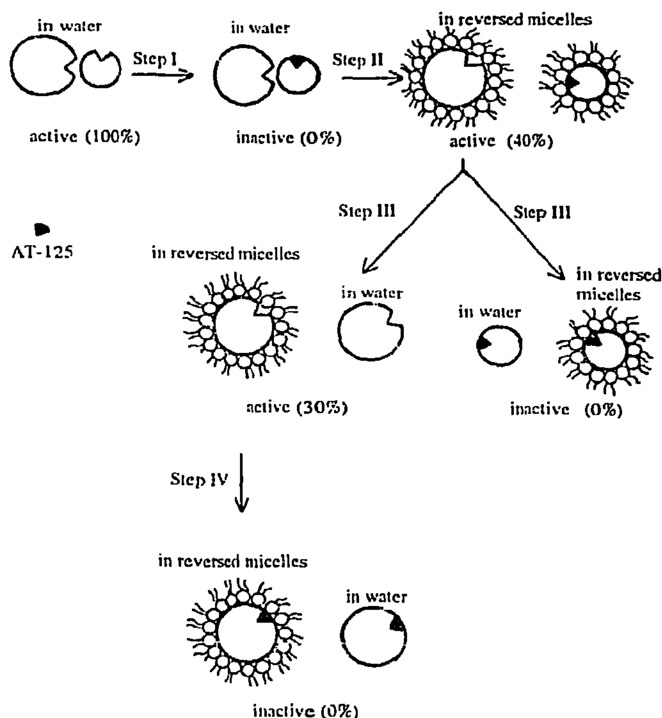

Fig. 2. The scheme of experiments on γ-GT inhibition with AT-125. Step I. The enzyme was incubated with AT-125 in aqueous solution and then purified from excess of inhibitor. The enzyme activity is not restored at least during its 10 h incubation in aqueous solution. Step II. Inactive enzyme obtained according to Step I is solubilized in AOT RM in octane ($w_0=17$). 5–10 min after solubilization, γ-GT activity is equal to 40% of the value measured under these conditions for the native enzyme. Step III. γ-GT subunits are separated by ultracentrifugation in the AOT RM system. Catalytic activity of subunits are studied either in micellar or aqueous solutions. Only the heavy subunit is active whereas the light one is inactive both in RM and aqueous solution. Step IV. The heavy γ-GT subunit is incubated in aqueous solution with AT-125 and purified as described for Step I. After such treatment the heavy subunit becomes inactive, its activity does not restore during incubation both in aqueous solution and in the AOT RM system. (Levels of enzyme activity given in brackets are expressed on a percentage basis of the values measured for the native γ-GT, corresponding to aqueous solution or the RM system.)

inactive (Fig. 2, Step III). The treatment of the isolated heavy subunit with AT-125 leads to its irreversible inactivation (Fig. 2, Step IV).

The data obtained indicate the presence of at least two active centers in the γ-GT molecule. The center located on the light subunit is accessible to the inhibitor when the enzyme is in a dimer form. The second center is masked under these conditions — no catalytic processing of the substrate molecule takes place and it is not accessible to the inhibitor in aqueous solution. Under the conditions of subunit dissociation in the RM system, the opening (remasking) of this center proceeds, resulting in a partial restoration of γ-GT activity. (It is highly probable that some change of the mutual orientation of the γ-GT subunits leading to the opening of the heavy subunit active center may proceed in RM even

under conditions when the enzyme represents a dimer. That may give an explanation to the appearance of the activity of the dimer observed in RM system (w_0=25) for AT-125-treated enzyme (see Fig. 1A, curve 2)). The fact that AT-125 inhibits both the light and the heavy γ-GT subunits indicates the structural similarity of both active centers.

Hence, the results of these studies permit to regard γ-GT as a multienzyme complex. The role of such a structure-function organization of this enzyme is not clear yet. It is known that γ-GT represents a membrane-bound enzyme [9]. Recently, a possible formation in biomembranes of non-bilayer lipid structures, built like the reversed micelles or closely resembling them (hexagonal phase H_{II}) received much attention in the literature [19-21]. Such structures may participate in the regulation of enzyme activity. It should be taken into consideration that the mechanism (analogous to that discussed in this paper) of the regulation of oligomeric composition and functional properties of the enzyme by changing the hydration degree and the geometry of the lipid matrices may also be important in living organisms [21].

REFERENCES

[1] Martinek, K., Levashov, A.V., Klyachko, N.L. and Berezin, I.V. (1977) Dokl. Acad. Nauk SSSR (in Russian) 236, 920-923 (Engl. edn.: DAN (1978) 236, 951-953).

[2] Klyachko, N.L., Merker, S., Vakulo, S.V., Ivanov, M.V., Berezin, I.V., Martinek, K. and Levashov, A.V. (1988) Dokl. Acad. Nauk SSSR (in Russian) 298, 1479-1481 (Engl. edn.: DAN (1988) 298, 56-58).

[3] Kabanov, A.V., Nametkin, S.N., Evtushenko, G.N., Chernov, N.N., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) Biochim. Biophys. Acta. 996, 147-152.

[4] Kabanov, A.V., Nametkin, S.N., Klyachko, N.L. and Levashov, A.V. (1991) FEBS Lett. 278, 143-146.

[5] Kabanov, A.V., Klyachko, N.L., Nametkin, S.N., Merker, S., Zaroza, A.V., Bunik, V.I., Ivanov, M.V. and Levashov, A.V. (1991) Protein Engineering 4, in press.

[6] Zulauf, M. and Eicke, H.F. (1979) J. Phys. Chem. 83, 480-486.

[7] Fletcher, P.D.I., Howe, A.M., Perrins, N.M., Robinson, B.H., Topracciogiu, C. and Dore, J.C. (1984) in: Surfactants in Solution (K.L. Mittal and B. Lindmann, eds.) vol. 2, Plenum, New York, pp. 1745-1753.

[8] Kabanov, A.V. (1991) Macromol. Chem. Macromol. Symp. 44, 253-264.

[9] Tate, S.S. and Meister, A. (1981) Mol. Cell. Biochem. 39, 1749-1781.

[10] Loginov, V.A., Chernov, N.N. and Berezov, T.T. (1980) Bull. Exp. Biol. (in Russian) 7, 58-60.

[11] Levashov, A.V., Khmelnitsky, Yu.L., Klyachko, N.L., Chernyak, V.Y. and Martinek, K. (1982) J. Colloid Interface Sci. 88, 444-457.

[12] Thompson, G.A. and Meister, A. (1976) Biochem. Biophys. Res. Commun. 71, 32-36.

[13] Martinek, K., Klyachko, N.L., Kabanov, A.V., Khmelnitsky, Yu.L. and Levashov, A.V. (1989) Biochim. Biophys. Acta 981, 161-172.

[14] Khmelnitskii, Yu.L., Kabanov, A.V., Klyachko, N.L., Levashov, A.V. and Martinek, K. (1989) in: Structure and Reactivity in Reverse Micelles (M.P. Pileni, ed.) Elsevier, Amsterdam, pp. 230-261.

[15] Gardell, S.J. and Tate, S.S. (1982) Arch. Biochem. Biophys. 216, 719-726.

[16] Tate, S.S. and Khadse, V. (1987) Arch. Biochem. Biophys. 255, 304-308.

[17] Reed, D.J., Ellis, W.W. and Meck, R.A. (1980) Biochem. Biophys. Res. Commun. 94, 1273-1277.

[18] Schasteen, C.S., Curtoys, N.P. and Reed, D.J. (1983) Biochem. Biophys. Res. Commun. 112, 564-570.

[19] Cullis, R.R., de Kruiff, B., Hope, M.S., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1983) in: Membrane Fluidity in Biology (R.C. Atoia, ed.) Academic Press, New York, pp. 39-81.

[20] De Kruijff, B. (1987) Nature, 329, 587-588.

[21] Mountford, C.E. and Wright, L.C. (1988) Trends Biochem. Sci. 13, 172-177.

[22] Klyachko, N.L., Levashov, Kabanov, A.V., Khmelnitskii, Yu.L. and Martinek, K. (1991) in: Kinetics and Catalysis in Microheterogeneous Systems (M. Gratzel and K. Kalyanasundaram, eds.) Marcel Dekker, New York, pp. 135-181.