# Serum IgG-Like Glycoferroprotein: Identification of Its Final Dissociation Form of Thermostable Protein Coupled with Albumin

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Human serum IgG-like glycoferroprotein identical to ascitic IgG-like glycoferroprotein that binds labeled monoclonal antibodies to CA125 is a complex consisting of three proteins: IgG, human serum albumin, and unidentified thermostable protein. Final dissociation form of serum IgG-like glycoferroprotein also appears as a complex of three nonidentical polypeptides with a molecular weight of 55 kDa (PC55) migrating in the albumin zone of thermostable protein coupled with albumin and structures chemically identical to human serum albumin and IgG heavy chains. Under denaturing conditions of electrophoresis in polyacrylamide gel, IgG-like glycoferroprotein and PC55 have the same molecular weight (about 55 kDa), while under reducing conditions their weight is about 75 kDa. Transition form (form the lower to the higher molecular weight) appears as an oblique (at about ~30o) protein band creating a ladder string effect. Ladder string effect was reproduced with thermostable protein coupled with albumin, PC55, IgG-like glycoferroprotein, with all commercially available human and bovine albumins, rat albumin as well as with heated and renatured albumins and can serve as electrophoretic identification sign for thermostable protein coupled with albumin. Renatured after boiling (100oC for 15 min) bovine albumin under reducing conditions appeared as bow string twisted in helix, that raises molecule in 2.5 turns from ~2 to ~75 kDa. These data attest to the existence of an albumin double and to its possible double structure.

**Key Words:** *serum IgG-like glycoferroprotein; CA125 antigen; thermostable protein linked to albumin; bow string effect; albumin helix*

Gamma-glycoferroprotein (GGFP) with a molecular weight of about 470 kDa and electrophoretic mobility of IgG was repeatedly detected in tumors and serum, but had different names: alfa-2H-globulin [6], alfa-2H-ferroglycoprotein [8], macromolecular serum ferroprotein, macromolecular albumin-IgG complex [13], CA125 antigen [4], macromolecular serum glycoprotein [2], tumor-associated IgG [14], new reactive protein [12], IgG-like serum structure [3], and peroxidase active glycoferroprotein, that binds labeled monoclonal antibodies (L-MAB) to CA125. However, in biochemistry this structure remains unknown.

Difficulties in its identification consist in that ascitic GGFP possessed not only the same electrophoretic mobility with IgG, but IgG was also one of the proteins in the GGFP complex, that also included human serum albumin (SA) and an unknown serum protein, *i.e.* GGFP precipitated with anti-IgG line identical to SA, and with antisera to human serum proteins like typical serum protein: absorption by donor serum inactivated the antiserum. GGFP was positively stained for glyco- and ferroproteins, possessed peroxidase activity (PA), and bound monoclonal antibodies (MAB) to CA125 antigen; IgG and CA did not exhibit these properties [12].

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Ascitic GGFP dissociated under conditions of non-denaturing electrophoresis in polyacrylamide gel (PAAG) with the formation of numerous intermediate forms, the main of which possessed electrophoretic mobility of α- and β-globulins with molecular weights of 230 kDa (A230) and 110 kDa (B110) [3]. However, the final GGFP dissociation form under denaturing conditions of SDS-PAAG electrophoresis appeared as a protein band with a molecular weight of 55 kDa, under reducing conditions it appeared also as a single band but with a molecular weight of 75 kDa [3,9]. The final GGFP dissociation form was also presented by a polypeptide complex with a molecular weight of 55 kDa (PC55) that included thermostable polypeptide and structures identical to SA and IgG heavy chain (IGHC).

In precipitation assay, PC55 interacted with polyclonal antisera to CA125 and to human serum proteins absorbed by SA and IGCH, but did not bind L-MAB to CA125. Under denaturing conditions of PAAG electrophoresis, PC55 migrated in the albumin zone and partially reassociated to a complex with a molecular weight of ~470 kDa that migrated at the start of 7.5% PAAG under denaturing conditions, was $Fe^{+3}$-positive, and exhibited PA. Similar phenomenon was observed in studies of alfa-2H-glycoprotein: renal tumor glycoprotein partially dissociated to albumin, which was not stained for glycoproteins, but albumin extracted from the renal tumor partially reassociated to glycoprotein in the IgG migration zone. These data on pulsating protein forms hit no explanation at that time and were not published; although later it was established that glycoprotein of alfa-2H-globulin pertains to human serum macroglobulins [2].

Thermostable protein-X appeared to be tightly coupled with albumin; separation from the albumin was failed even after heating (100°C for 30 min), and it was called thermostable protein coupled with albumin. Double complex thermostable protein coupled with albumin–albumin was detected using precipitation assay in ascitic GGFP and in all its isoforms including the final one [5]. Therefore, albumin complexes and fragments as well as IgG can be detected in any zone of electrophoregram and can have different molecular weights. For instance, SA-IgG complex was described [13], 8 SA complexes and fragments with molecular weights of 210, 168, 147, 132, 110, 45, 28, and 19 kDa were detected in human plasma [10], 9 structures with molecular weights 260, 180, 110, 45, 40, 30, 24, 18, and 11 kDa were detected in the urine [15], and IgG curve in immunoelectrophoresis can cover all zones including SA migration zone.

This article presents the key results of 10-year investigations of serum IgG-like GGFP serum protein and identification of its final dissociation form, thermostable protein coupled with albumin.

## MATERIALS AND METHODS

CA125 antigen was isolated from acid-soluble ascitis fraction and pooled serum obtained from female donors according to Davis. Level of immunoreactive CA125 was determined using electrochemiluminescence assay and Hossmann-L Roche Elecsys 2010 device as well as by ELISA with L-MAB to CA125 antigen. Blood serum with immunoreactive CA125 content of 34.6 U/ml or 5 μg/ml and ascitic fluid with protein level of 3140 U/ml or 448 μg/ml were used as the control. Standard samples included CA125 antigen extracted according to Davis from blood serum from 50 female donors – 63.6 U/mg, serum of patient B. (287 U/ml), GGFP 470 (48.3 U/mg), A230 (514 U/mg), PC55 (1.6 U/mg), and IgG (0.02 U/mg). Ascitic fluid without hemolysis was used.

GGFP 500 from donor serum was obtained by gel-filtration of serum fraction insoluble at 30% saturation with ammonium sulfate, with subsequent selection of fractions with a molecular weight of ~500 kDa. PC55 (albumin zone) was isolated donor serum diluted 1:8 by native PAAG in tubes. Thermostable complex thermostable protein coupled with albumin–albumin was obtained after heating albumin preparations (2-10 mg/ml; at 100°C for 15 min). All GGFP isoforms from ascitic fluid were isolated *ex tempore* because of extremely rapid changeability of the forms.

Fraction with a molecular weight of 55-60 kDa was isolated from IgG (2 mg/ml) using gel-filtration under the control of precipitation test-system on IGHC.

SA or BSA (Sigma) 2-10 mg/ml were dissolved in Tris-glycine buffer (0.5 mM Tris, 0.03% glycine pH 8.3) and incubated for 15 min in a boiling water bath; denatured protein was separated by centrifugation, suspended in Tris-glycine buffer (0.05 M Tris, 3% glycine pH 8.3), and renatured for 20 h at 4°C (about 0.5% protein is renatured).

The study was carried out using only commercial samples of polyclonal antisera to human serum proteins (Gamaleya Scientific Research Institute of Epidemiology and Microbiology; Binding Site Limited, Sigma), anti-IgG (Gamaleya Scientific Research Institute of Epidemiology and Microbiology) and antiserum to γ-, α-, and μ-chains of IgG, IgA, IgM (Bio-Rad). Rabbit antisera to GGFP and PC55 were obtained by us.

After native PAAG, plates/columns were incubated with commercial L-MAB (to CA125 and to α-fetoprotein) and labeled polyclonal antibodies to trophoblastic β-glycoprotein for ELISA for 1 h at 37°C and 16 h at 4°C, three times washed with water for 15 min, incubated (37°C for 15 min) with 3,3,5,5-tetramethyl benzidine, and washed with water. Another

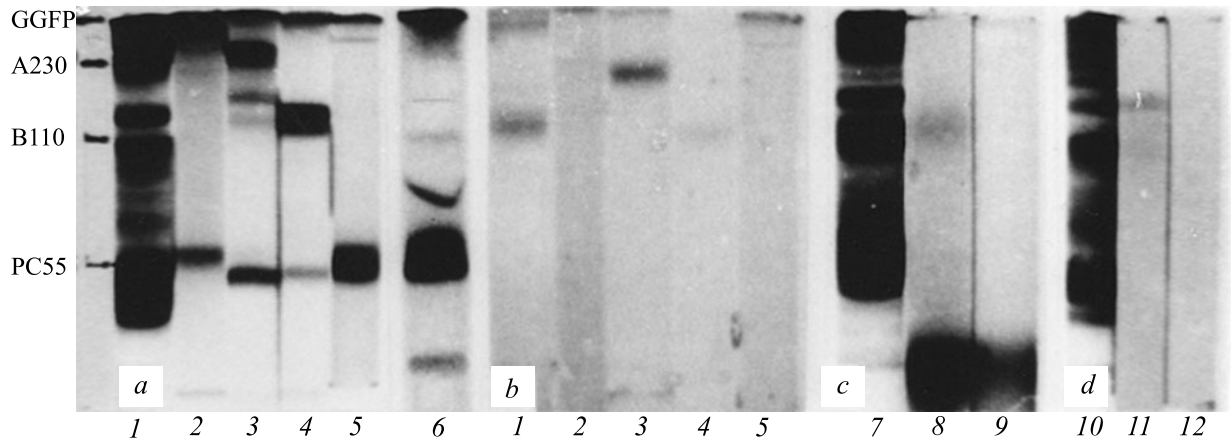


**Fig. 1.** Electrophoretic characteristics of GGFP and its isoforms in non-denaturing PAAG electrophoresis in tubes. *a*: electrophoretic mobility of GGFP and its isoforms in 7.5% native PAAG: *1*) ascites, ovarian cancer patient № 305; *2*) GGFP; *3*) A230; *4*) B110, *5*) PC55, isolated *ex tempore* according to Davis (electrophoresis dated 26.07.2005); *6*) dynamics of PC55 reassociation 7 month later (electrophoresis dated 03.03.2006). Protein staining. *b*: PA of ascites 305, GGFP and it isoforms with substrate DAB; *c*: direct binding of L-MAB to CA125 with antigen of blood serum from patient B. in 7.5% PAAG. *7*) protein staining; *8*) DAB staining development after incubation with L-MAB to CA125; *9*) DAB staining development; *d*: direct binding of L-MAB to CA125 with antigen of acsites No. 307. *10*) protein staining; staining development with substrate 3,3,5,5-tetramethyl benzidine after incubation with L-MAB to CA125; *12*) staining development with substrate 3,3,5,5-tetramethyl benzidine.

substrate (3,3-diaminobenzidine tetrahydrochloride, DAB) was established to oxidize all isoforms of ascitic CA125, excluding the final one, without preliminary incubation with L-MAB (Fig.1, *b*).

PAAG plates/columns after electrophoresis were incubated in DAB (Sigma) solution with hydrogen peroxide (DAB 10 mg was dissolved *ex tempore* in 20 ml 0.05 M Tris-HCl buffer, pH 7.5, with addition of 0.3 ml 3% $H_2O_2$) 15-30 min at 37°C and washed with water 16-18 h at 4°C. To control PA specificity of CA125 isoforms, we used SA, α-fetoprotein, and trophoblastic β-glycoprotein that do not oxidize DAB.

Protein level was determined spectrophotometrically at λ=260 and 280 нм.

Protein staining was carried out in 0.25% Coomassi G-250 in ethanol--acetic acid--water 5:1:4 mixture for 15 min followed by decoloration in water on a boiling water bath for 15-30 min.

PAAG plates/columns were incubated for 16-20 h in a mixture of 3% $K_4[Fe(CN)_6]\times 3H_2O$ and 10% HCl (ratio1:1 *ex tempore*) and washed in 5% HCl [2]. $Fe^{+3}$ is weakly bound in GGFP and its isoforms and after heating it was virtually undetectable.

Glycoproteins were assayed by Schick reaction [3].

Direct precipitation assay with mouse L-MAB and antiserum to human serum proteins was performed: L-MAB acted as antigen in this assay. The method is based on the cross-reactivity between human and mouse IgG [1]. To detect mouse monoclonal antibodies, any commercially available antiserum to human serum proteins (or anti-IgG) was used, and band of precipitation with L-MAB was stained with the substrate (Fig. 2, *d*).

## RESULTS

High mobility of dissociation-association processes of GGFP and its isoforms is most pronounced in ascitis (Fig. 1, *a*): on the one hand, GGFP, A230, and B110 partially dissociate down to the final form, PC55 (Fig. 1, *a*, rows *2-4*), on the other hand, PC55 partially reassociates to GGFP (Fig. 1, *a*, rows *5* and *6*), which reflects pulsating form of protein body existence, the property characteristic for many enzymes. PA of ascitic GGFP isoforms before and after incubation with L-MAB to CA125 was the same (Fig. 1, *b*). GGFP final dissociation form PC55 possessed no PA, but PC55 that partially reassociated to GGFP exhibited PA (Fig. 1, *b*, row *5*). Seven month later, the same form, PC55, under conditions of native PAAG in tubes yielded up to 6 protein bands, one of which was oblique (Fig. 1, *a*, row *6*). Similar band was also observed for final dissociation form of ascitic CA125 with a molecular weight of 55 kDa, but under reducing conditions on PAAG plate [9].

In serum analysis we first reproduced and confirmed the results obtained in 1970s: donor macroglobulin in the IgG migration zone was constantly stained for ferro- and glycoproteins [2]. Furthermore, GGFP protein with PA that was stained for $Fe^{+3}$ was detected in human, bovine, and rat albumin samples isolated from the serum *ex tempore*; IgG possessed no such properties and was not identical to ascitic and serum GGFP (Fig. 2, *a*). Direct binding of L-MAB to CA125 with antigen after native PAAG electrophoresis of patient's serum (287 U/ml of immunoreactive CA125) was observed only with B110 form, but in contrast to

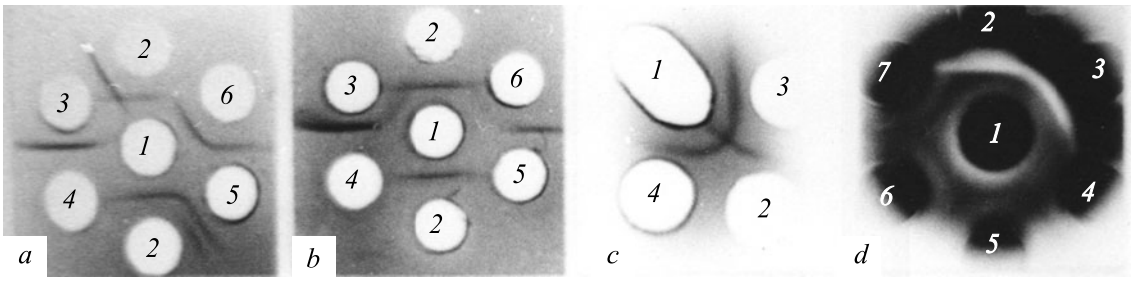


**Fig. 2.** Identification of GGFP, thermostable protein coupled with albumin, and determination of L-MAB using direct precipitation assay. *a*: comparison of test-systems on human GGFP and IgG: *1*) antiserum to ascites GGFP, depleted by IgG; *2*) serum GGFP; *3*) anti-IgG; *4*) IgG; *5*) antiserum to human serum proteins, depleted by SA; *6*) ascites GGFP. *b*: comparison of test-systems on thermostable protein coupled with albumin, human SA and IgG: *1*) antithermostable protein coupled with albumin (for illustration purposes, overdepleted by IgG and subdepleted by SA); *2*) thermostable protein coupled with albumin; *3*) anti-IgG; *4*) IgG; *5*) anti-SA; *6*) SA. *c*: comparison of test-system on thermostable protein coupled with albumin and IGHC: *1*) antithermostable protein coupled with albumin; *2*) thermostable protein coupled with albumin; *3*) antiserum to IgG, IgA, IgM heavy chains; *4*) IGHC. *d*: identification of mouse L-MAB in direct precipitation assay with antiserum to human serum proteins: *1*) L-MAB to CA125; *2*) whole commercial antiserum to human serum proteins; *3-7*) antisera in the ratio of 1:2, 1:8, 1:32, 1:128, and 1:512. Staining development by substrate DAB.

ascitic CA125 form it possessed no PA with DAB (Fig. 1, *c*). In ascitic fluid, 1-2 (rarely 3) protein forms bind L-MAB to CA125 (Fig. 1, *d*, row *11*).

Further serum investigations were focused on albumins. Single protein band with a molecular weight of ~55 kDa was observed in this zone after complete GGFP dissociation in denaturing PAAG (Fig. 3, *a*). The protein identical to ascitic thermostable protein coupled with albumin was detected in all donor's serum samples and SA preparations (Fig. 2, *a*), and its level in donor's serum was about 0.01% serum protein, in SA preparations it was up to 0.1%, and after SA heating it was up to 1.5%. It should be noted that thermostable protein coupled with albumin interacts only with polyclonal antisera. Thermostable protein coupled with albumin was established to be immunochemically identical to SA, IgG, and IGHC (Fig. 2, *b*, *c*), but always appear with albumin and disguised as albumin even more than GGFP disguised as IgG.

GGFP and PC55 appear in denaturing PAAG as one protein band with a molecular weight of ~55 kDa; in reducing PAAG they appear also as one band but with a molecular weight of ~75 kDa, whereas in IgG as two bands with molecular weights of about 55 and 12 kDa; band with a molecular weight of 75 kDa is absent (Fig. 3, *a*, rows *2-14*). Molecular weight of GGFP and thermostable protein coupled with albumin before and after reduction of SS-bounds is the same

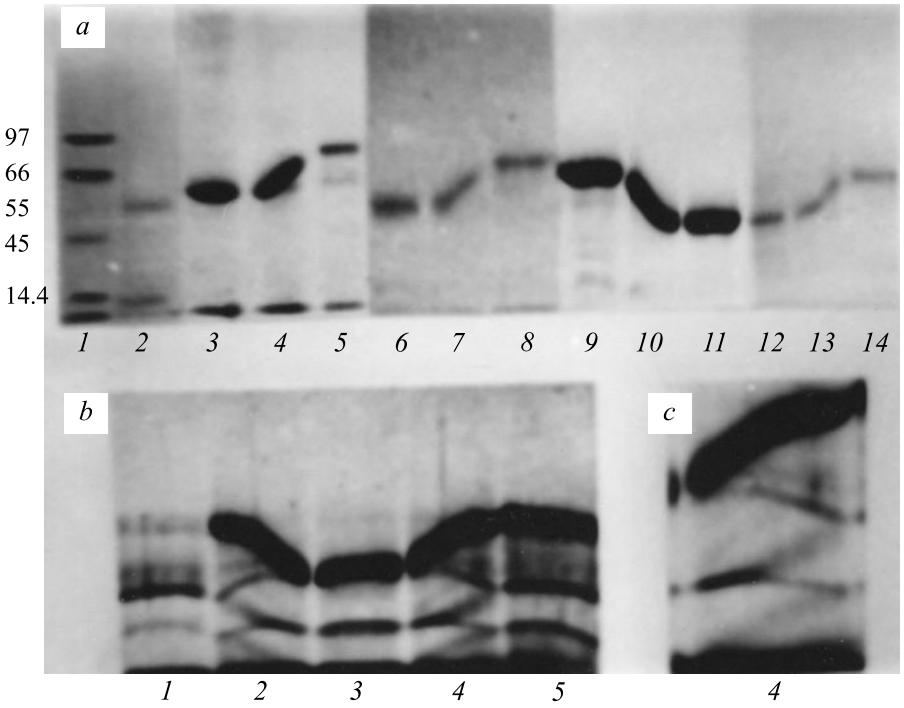


**Fig. 3.** Electrophoresis of serum proteins in 10% PAAG plates under denaturing conditions. *a*: bowstring effect: *1*) molecular weight markers (14.4-97 kDa); *2*) IgG; *3-5*) GGFP; *6-8*) serum PC55; *9-11*) BSA; *12-14*) rat albumin. Rows *1, 2, 5, 8, 9, 14* contain 2% SDS and 5% mercaptoethanol (ME); rows *3, 4, 6, 7, 10-13* contain 2% SDS. *b*: albumin spiral: *1*) thermostable BSA; *2-5*) renatured BSA (in rows *2* and *4* – albumin spiral). Rows *1*, *5* contain 2% SDS and 5% ME; *2-4*) 2% SDS. *c*: albumin spiral (3.5-fold magnification).

and increases only by 20 kDa, which was invariably demonstrated by SA, BSA and rat albumin (Fig. 3, *a*, rows *6-14*), as well as by heated and renaturized BSA (Fig. 3, *b*) and SA. This was not observed for IgG (Fig. 3, *a*, row *2*) and α-fetoprotein.

Transitional form from lower to higher molecular weight of the structure appears as oblique (at about 30°) band connecting levels with different values (Fig. 3, *a*). There were no expected numerous horizontal bands; thus, ladder string covers a number of horizontal bands by one oblique band. Bowstring effect was reproduced with thermostable protein coupled with albumin, PC55, GGFP, and all native, heated and renatured SA and BSA preparations, as well as with rat albumin and apparently appear as the key moment in reconstruction the functional structures from stem complex thermostable protein coupled with albumin–albumin (~1:30). The formation bowstring by this complex in native PAAG (Fig. 1, *a*, row *6*), *i.e.* in the absence of exogenous thiols, at first sight indicates mercaptalbumin involvement in the process, however, cysteine density in α-fetoprotein and IGHC is higher than in SA, but they do not form bowstring even with mercaptoethanol. In renatured BSA, bowstring line raises in closed spiral mode from approximately 2 to 75 kDa (Fig. 3, *b*, pats *2* and *4*), while "shadow" of the second spiral at higher levels (Fig. 3, *b*, rows *2* and *4*) does not exclude its duplex nature.

Abundance of thermostable protein antigen coupled with albumin is confirmed by its detection by precipitation assay in all serum samples and commercially available human preparations: SA, IgG, and in antiserum to IGHC (Fig. 2, *c*), i.e. where SA was detected: the depot of thermostable protein coupled with albumin. Bowstring effect observed with BSA and rat albumin is indicative for the functionally similar structure in the animals. Antibodies to thermostable protein couples with albumin were detected in all commercial antisera to human serum proteins in the titer of 1:10 (Sigma) and in the titer up to 1:40 (Binding Site Limited; Gamaleya Scientific Research Institute of Epidemiology and Microbiology).

We assume that primary structure of thermostable protein coupled with albumin appears in extracellular N-end domain of CA125 (1-1638 b.p., contains no cysteine) and doubled in peptide motifs: 421-524 b.p. and 641-742 b.p. [11]. The complex of termostable protein coupled with albumin–albumin is apparently the stem structure of all CA25 structures up to super complexes with a molecular weight of 2700 kDa [10]. Their number and content apparently depends on the content of free/toxic degradation products in cells (iron ions, carbohydrates, hem-groups etc.), which is clearly seen in ascitic fluid of ovary cancer patients. Identification of thermostable protein coupled with albumin implies double structure of albumin, because the nature of pulsating form of protein existence, reversible molecule stretching, heterogeneity, acquisition of enzymatic activity by reassociated forms, and reconstruction of denaturized protein using spiral principle cannot be explained by single configuration adaptability of monovalent albumin structure, and it is of interest for further investigations.

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