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Formation of an intramolecular disulfide bond of glycolipid transfer protein

Akira Abe and Terukatsu Sasaki

Department of Biochemistry, Sapporo Medical College, Sapporo (Japan)

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Functional and structural differences between the two forms of glycolipid transfer protein (GLTP) with (the faster component) and without (the slower component) an intramolecular disulfide bond were studied. GLTP treated with *N*-ethylmaleimide (NEM) and $Na_2S_4O_6$ had a transfer activity of about 70% and 55%, respectively, of the control GLTP. No significant decrease was found in the binding affinity of NEM-treated GLTP to pyrene-labeled galactosylceramide (PyrGalCer). A small decrease in the binding affinity was found in the $Na_2S_4O_6$-treated GLTP. Oxidation of NEM-treated and $Na_2S_4O_6$-treated GLTP catalyzed by $CuSO_4$ resulted in a stoichiometric conversion of the slower component to the faster component. The faster component thus formed was quantitatively reduced back to the slower component by treatment with 2-mercaptoethanol in the presence of 1% SDS. These results provided strong evidence for the conversion of the slower component to the faster component as a result of the formation of an intramolecular disulfide bond. The transfer activity of the NEM-treated and oxidized GLTP (the faster component) was 1.7-fold higher than that of the original GLTP and 2.4-fold higher than that of the NEM-treated GLTP. The transfer activity of the $Na_2S_4O_6$-treated and oxidized GLTP was 2-fold higher than that of the original GLTP and 3.6-fold higher than that of the $Na_2S_4O_6$-treated GLTP. The binding affinity of the faster components, produced from both the NEM-treated GLTP and the $Na_2S_4O_6$-treated GLTP, to PyrGalCer was found to be twice that of the respective modified GLTPs before oxidation. By circular dichroism measurements, it was found that a small decrease in the magnitude of mean residue ellipticity but no significant change in ellipticity spectrum was brought about either upon the modification of GLTP by $Na_2S_4O_6$ or upon the formation of an intramolecular disulfide bond in GLTP. The results suggest that the formation of an intramolecular disulfide bond results in only a small change in the secondary and tertiary structure of GLTP. The results presented in this paper suggest that the transfer activity of GLTP may be regulated by the redox state of sulfhydryl groups present in GLTP.

Introduction

In the preceding paper (Ref. 11), we reported that GLTP purified from pig brain is a mixture of two forms of a protein with (about 15%) and without (about 80%) an intramolecular disulfide bond. The results presented in that paper [11] also indicate that GLTP has one additional sulfhydryl group available for reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and *N*-ethylmaleimide (NEM) under nondenaturing conditions.

It is well known that activities of some enzymes and receptors are modulated by the formation of intramolecular disulfide bonds and by the covalent modification of free sulfhydryl groups [1,2]. It is possible that the transfer reaction of glycolipids facilitated by GLTP is regulated by the formation and cleavage of an intramolecular disulfide bond in the GLTP molecule. In this paper, we investigated functional and structural differences between the two forms of GLTP with and without an intramolecular disulfide bond.

Materials and Methods

Preparation of GLTP. GLTP was purified from pig brain as described previously [3,11]. Purified GLTP gave a single band with an estimated molecular mass 22 kDa on SDS-PAGE under reducing conditions according to the method of Laemmli and Favre [4]. Protein

Abbreviations: GalCer, galactosylceramide; PyrGalCer, 1-*O*-(β-D-galactopyranosyl)-*N*-[10-(1-pyrenyl)decanoyl]-D-*erythro*-sphingosine; GLTP, glycolipid transfer protein; *E*/*M* ratio, excimer/excited monomer emission intensity ratio.

Correspondence: A. Abe, Department of Biochemistry, Sapporo Medical College, South-1, West-17, Sapporo 060, Japan.

was determined by the method of Bensadoun and Weinstein [5].

GLTP activity was determined by the fluorimetric assay [6].

Preparation of GLTP containing an intramolecular disulfide bond from GLTP treated with NEM. Purified GLTP was dialyzed against 150 mM NaCl, 20 mM Tris-HCl, pH 7. After dialysis, GLTP (143 μg) was incubated with 0.45 mM NEM for 30 min at 20 °C. The incubation mixture was applied to a PD-10 column (Pharmacia). The chromatography was carried out in the same buffer at a flow rate of about 1 ml per min at 20 °C. Fractions of 1 ml were collected. GLTP (124 μg) was recovered in fractions 4 to 6. NEM-treated GLTP was incubated with 1 mM $CuSO_4$. Incubation was carried out in a total volume 100–250 μl in a polyallomer tube (9 × 60 mm) covered with Parafilm under shaking for 30 min at 20 °C. The reaction was stopped by the addition of 6 mM EDTA. Each sample was stored in a polyallomer tube at 4 °C.

Preparation of GLTP containing an intramolecular disulfide bond from GLTP treated with sodium tetrathionate. Tetrathionate ($S_4O_6^{2-}$) reacts selectively with the sulfhydryl group, resulting in the formation of a sulfenylthiosulfate derivative, from which the original sulfhydryl group is readily regenerated by the addition of thiol reagents.

Purified GLTP (400 μg) was incubated with 2 mM $Na_2S_4O_6$, with shaking for 30 min at 20 °C. The incubation mixture (3 ml) was dialyzed against 150 mM NaCl, 20 mM Tris-HCl, pH 7.4. Dialysis was carried out at 4 °C. $Na_2S_4O_6$-treated GLTP was incubated with 500 μM $CuSO_4$. Incubation was carried out in a total volume 1.5 ml in a polyallomer tube (9 × 60 mm) covered with Parafilm, with shaking for 90 min at 20 °C. Each GLTP, the control GLTP, $Na_2S_4O_6$-treated GLTP and $Na_2S_4O_6$/$CuSO_4$-treated GLTP, was dialyzed against 50 mM NaCl, 5 mM sodium phosphate (pH 7.3) at 4 °C. After dialysis, each sample was stored in a polyallomer tube at 4 °C.

Circular dichroism of GLTP. Circular dichroism (CD) spectra were measured at 25 °C using a J-500 spectropolarimeter (JASCO, Tokyo). Scans were carried out with a scale sensitivity of 2 m°/cm and a time constant of 1 s. Solution for CD were below 100 μg/ml of protein in 50 mM NaCl, 5 mM sodium phosphate (pH 7.3). The concentrations of the control GLTP, $Na_2S_4O_6$-treated GLTP and $Na_2S_4O_6$/$CuSO_4$-treated GLTP were 94.3 μg/ml, 93.2 μg/ml and 69.3 μg/ml, respectively.

Results

In the preceding paper [11], it has been shown that the oxidation of GLTP catalyzed by Cu^{2+} produces

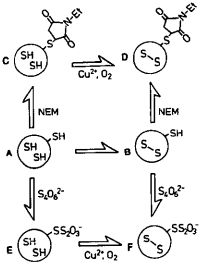

Fig. 1. The pathway of disulfide formation in GLTP. Purified GLTP from pig brain is a mixture of two forms of a protein with (about 15%, form B) and without (about 80%, form A) an intramolecular disulfide bond. The A → B reaction is an oxidation process but in vitro, it is not easy to produce form B from form A without masking the sulfhydryl group reactive with thiol reagents. (C) The slower component modified with NEM. (D) The NEM-faster component modified with NEM. (E) The slower component modified with $S_4O_6^{2-}$. (F) The faster component modified with $S_4O_6^{2-}$.

oligomeric GLTPs and GLTP with an intramolecular disulfide bond (the faster component). The oligomeric GLTPs are produced as a result of the formation of intermolecular disulfide bonds between free sulfhydryl groups of GLTP, one of which is reactive with DTNB under nondenaturing conditions and is important for the formation of the intermolecular disulfide bond [11]. Moreover, it has been suggested that the faster component (form B in Fig. 1) of GLTP containing a natural intramolecular disulfide bond is responsible for most of the transfer activity in GLTP. In order to investigate the structural and functional differences between GLTPs without (form A) and with (form B) an intramolecular disulfide bond, it was necessary to find a condition under which the slower component could be transformed to the faster component without concomitant formation of oligomeric GLTPs. It has been difficult to find such a condition because oligomeric GLTPs are formed upon exposure of GLTP to oxidizing conditions. Therefore, the reactive sulfhydryl group on the surface of GLTP was masked either by NEM or by sodium tetrathionate, $Na_2S_4O_6$, before introducing an intramolecular disulfide bond.

It was not successful to transform the faster component to the slower component by reduction with 2-mercaptoethanol or dithiothreitol under nondenaturing conditions. Therefore, GLTP consisting of a mixture of about 80% of the slower component (form A) and about 15% of the faster component (form B) was used as the control GLTP.

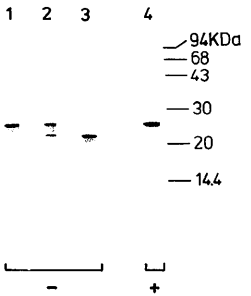

Fig. 2. Formation of the faster component of GLTP by treatment with NEM and $CuSO_4$. NEM-treated GLTP was incubated with 1 mM $CuSO_4$ as described under Materials and Methods and analyzed by SDS-PAGE according to the method of Laemmli and Favre [4]. Samples for SDS-PAGE contained 3.6 μg protein per lane, 1% SDS, 0.005% Bromophenol blue and 2.5% 2-mercaptoethanol (2-ME) where indicated. The samples were not boiled. Lane 1, GLTP without NEM-treatment; lanes 2, 3 and 4, NEM-treated GLTP. Lanes 3, 4 were treated with 1 mM $CuSO_4$.

Preparation of GLTP containing an intramolecular disulfide bond from GLTP treated with NEM

GLTP purified from pig brain was incubated with 0.45 mM NEM for 30 min at 20 °C, after which GLTP was separated from excess NEM by chromatography on a PD-10 (Pharmacia) column. As a control, GLTP was treated under the same conditions in the absence of NEM. Control and NEM-treated GLTP was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 2). It was estimated by densitometry that in GLTP incubated in the absence of NEM, the slower component (form A) and the faster component (form B) accounted for about 80% and about 15%, respectively, of the total protein. Incubation of GLTP with NEM resulted in a slight increase in the faster component modified with NEM (NEM-faster component) (Fig. 2) and in a decrease in transfer activity to about 70% of the control level (data not shown). This partial inactivation by NEM had previously been observed [6].

Oxidation of NEM-treated GLTP catalyzed by 1 mM $CuSO_4$ resulted in a stoichiometric conversion of the slower component modified with NEM (NEM-slower component) to the NEM-faster component (Fig. 2). The NEM-faster component was quantitatively converted to the NEM-slower component by reduction with 2.5% 2-mercaptoethanol in the presence of 1% SDS (Fig. 2). Reduction of the NEM-faster component with 2-mercaptoethanol under nondenaturing conditions partially converted the component to the NEM-slower component (data not shown). It was found that the $CuSO_4$-catalyzed conversion of the NEM-slower component to the NEM-faster component resulted in an increase of transfer activity by 1.7-fold when compared with original GLTP and by 2.4-fold when compared with NEM-treated GLTP (data not shown). When $CuSO_4$ concentration added in the oxidation was reduced from 1 mM to 0.1 mM, the NEM-slower component was not completely converted to the NEM-faster component (data not shown).

Binding of PyrGalCer to NEM-treated and oxidized GLTP (NEM-faster component)

The formation of a complex between GLTP and PyrGalCer can be determined by fluorescence measurements of a mixture of GLTP and phosphatidylcholine vesicles containing PyrGalCer [6]. A decrease in the eximer emission intensity (E)/excited monomer emission intensity (M) ratio is a measure of the formation of the GLTP-PyrGalCer complex formation. The magnitude of change in the E/M ratio correlates with the amount of GLTP-PyrGalCer complex formed or with the binding affinity of GLTP to PyrGalCer when the amount of GLTP is kept constant [6].

The change in the E/M ratio produced by the addition of 5.56 μg GLTP to the vesicles containing 0.38 nmol PyrGalCer was compared oetween control GLTP, NEM-treated GLTP, and NEM-treated and oxidized GLTP (the NEM-faster component) (data not shown). There was no significant difference between control GLTP and NEM-treated GLTP in this intensity ratio. However, about a 2-fold decrease in E/M ratio was observed by the addition of the NEM-faster component, which indicates a 2-fold increase in the binding affinity of the modified GLTP to PyrGalCer.

Preparation of GLTP containing an intramolecular disulfide bond from GLTP treated with sodium tetrathionate

$Na_2S_4O_6$ reacts selectively with the sulfhydryl group of a protein without significant reaction with other amino acid residues of the protein. GLTP was incubated with 2 mM $Na_2S_4O_6$ for 30 min at 20 °C, after which excess $Na_2S_4O_6$ was removed from the reaction mixture by dialysis against 0.15 M NaCl, 20 mM Tris-HCl, pH 7.4. As a control, GLTP was treated under the same conditions except in the absence of $Na_2S_4O_6$. Analysis by SDS-PAGE under nonreducing conditions revealed a slight decrease of the *S*-sulfenylsulfo-faster component (SS-faster component) in $Na_2S_4O_6$-treated GLTP (Fig. 3). Incubation of GLTP with $Na_2S_4O_6$ resulted in a decrease in the transfer activity to about 55% of the control level (data not shown). The transfer activity of $Na_2S_4O_6$-treated GLTP was restored to the original level by incubation with 33 mM DTT (data not shown).

When $Na_2S_4O_6$-treated GLTP was incubated with 500 μM $CuSO_4$ for 90 min at 20 °C, the *S*-

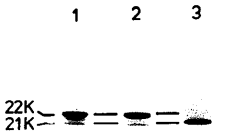

Fig. 3. Formation of the faster component of GLTP by treatment with $Na_2S_4O_6$ and $CuSO_4$. $Na_2S_4O_6$-treated GLTP were incubated with 500 μM $CuSO_4$ as described under Materials and Methods and analyzed by SDS-PAGE according to the method of Laemmli and Favre [4]. Samples for SDS-PAGE contained 3.6 μg protein per lane, 1% SDS. 0.005% Bromophenol blue. The samples were not boiled. Lane 1, control GLTP; lane 2, $Na_2S_4O_6$-treated GLTP; lane 3, $Na_2S_4O_6$-treated and $CuSO_4$-oxidized GLTP. Reduction of each SDS-sample by 2-mercaptoethanol resulted in the conversion of the faster component to slower component (data not shown). GLTP preparations were obtained as described under Materials and Methods.

sulfenylsulfo-slower component (SS-slower component) was stoichiometrically converted to the SS-faster component (Fig. 3). Decreasing $CuSO_4$ concentration from 500 μM to 50 μM resulted in an incomplete conversion of the SS-slower component to the SS-faster component (data not shown). Treatment of the SS-faster component with 1% 2-mercaptoethanol in the presence of 1% SDS quantitatively converted the component to the SS-slower component (data not shown). Treatment of

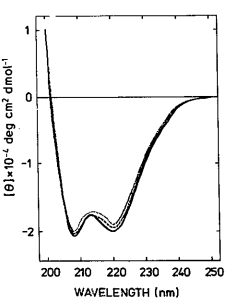

Fig. 4. Circular dichroism spectra of control GLTP (———), $Na_2S_4O_6$-treated GLTP (— — —) and $Na_2S_4O_6$-treated and oxidized GLTP (·····) in 50 mM NaCl, 5 mM sodium phosphate, pH 7.3. Circular dichroism spectrum of each GLTP preparation was measured as described under Materials and Methods.

TABLE I

The percentages of α-helix and β-structure of GLTP by CD method

The assay was carried out as described under Materials and Methods. The values were based on CD spectra between 202 and 240 nm in Fig. 4 according to the method of Chen et al. [7].

GLTP	α-Helix	β-Structure
Control	60.2	25.3
$Na_2S_4O_6$-treatment	58.5	29.5
$Na_2S_4O_6$ and $CuSO_4$-treatment	55.6	26.7

the SS-faster component with 2% 2-mercaptoethanol under non-denaturing conditions partially converted the component to the SS-slower component. It was found that the $CuSO_4$-catalyzed conversion of the SS-slower component to the SS-faster component resulted in an increase in the transfer activity by 2-fold when compared with the original GLTP and by 3.6-fold when compared with the $Na_2S_4O_6$-treated GLTP (data not shown).

Binding of PyrGalCer to $Na_2S_4O_6$-treated and oxidized GLTP (SS-faster component)

The change in the E/M ratio produced by the addition of about 6 μg GLTP to the vesicles containing 0.38 nmol PyrGalCer was compared (data not shown). The decrease in E/M ratio produced by the addition of $Na_2S_4O_6$-treated GLTP was smaller than that produced by control GLTP. The $Na_2S_4O_6$-treated and oxidized GLTP (the SS faster component) produced a 2-fold larger decrease of E/M ratio than the $Na_2S_4O_6$-treated GLTP, which again indicates a 2-fold increase in the binding affinity of the modified GLTP to PyrGalCer.

CD spectrum of GLTP

CD spectra of control GLTP, $Na_2S_4O_6$-treated GLTP, and $Na_2S_4O_6$-treated and oxidized GLTP (the SS-faster component) are shown in Fig. 4. Each of GLTPs exhibited two ellipticity bands at 208 nm and 220 nm indicating the presence of an α-helix. The $Na_2S_4O_6$-treated GLTP showed a decrease in the magnitude of mean residue ellipticity. This decrease became a little more obvious after the oxidation of the treated GLTP in the presence of Cu^{2+}.

The α-helix and β-structure contents of GLTP were estimated by the method of Chen et al. (Table I) [7]. β-Structure content increased by 16% upon treatment of GLTP with $Na_2S_4O_6$. α-Helix content decreased by 10% upon the $CuSO_4$-catalyzed oxidation of $Na_2S_4O_6$-treated GLTP.

Discussion

This paper described the formation of the faster component of GLTP from the slower component by

introducing an intramolecular disulfide bond (Fig. 1). Thus, the relationship between two components of GLTP present in purified GLTP was unambiguously determined. In the first experiment, GLTP was modified with NEM which specifically reacts with sulfhydryl group during a short period of time in neutral pH. The NEM-faster component (form D in Fig. 1) was formed from the NEM-slower component (form C in Fig. 1) by Cu^{2+}-catalyzed oxidation or from the faster component (from B in Fig. 1) by NEM-treatment. Form D thus formed was converted to the NEM-slower component (form C in Fig. 1) by reduction with 2-mercaptoethanol in SDS. The sulfhydryl group in GLTP modified with NEM is most likely identical with the sulfhydryl group reactive with DTNB under nondenaturing conditions, which was described in the preceding paper [11]. Therefore, the production of form D by introducing an intramolecular disulfide bond in form C supports the conclusion drawn in the preceding paper that the slower component (form A in Fig. 1) contains three sulfhydryl groups in a molecule and that one of them exists on the surface of the molecule.

Treatment of GLTP with NEM resulted in a partial inhibition of the transfer activity. The transformation of form C to form D resulted in an activation of the transfer activity of GLTP. This suggests that the transfer activity of GLTP can be modulated by the formation and cleavage of an intramolecular disulfide bond.

In the PyrGalCer binding assay of GLTP, no difference was found in the apparent binding affinity between the control and NEM-treated GLTP. Therefore, the difference in transfer activity between the control and NEM-treated GLTP probably originates from a difference in the rate of formation and decomposition of the GLTP-PyrGalCer complex. On the other hand, it was found that the binding affinity of form D to PyrGalCer was twice of that of control GLTP. Therefore, about 2-fold enhancement of transfer activity found as a consequence of formation of an intramolecular disulfide bond can be explained by an increase in the affinity of GLTP to PyrGalCer. An increase in the rate of formation and decomposition of the GLTP-PyrGalCer complex may also result from the formation of an intramolecular disulfide bond.

In some cases, the treatment of proteins with $S_4O_6^{2-}$ results in the formation of an intramolecular disulfide bond [8], which is thought to be formed by the reaction of the sulfenylthiosulfate derivative with a proximate sulfhydryl group [9]. The results obtained by the use of $Na_2S_4O_6$ in place of NEM were very similar to those obtained by the use of NEM, and could be summarized as follows; (1) GLTP contains a sulfhydryl group sensitive to $S_4O_6^{2-}$, which is not located in the proximity of the sulfhydryl group involved in the formation of an intramolecular disulfide bond, (2) the transformation of the SS-slower component (form E in Fig. 1) to the SS-faster component (form F in Fig. 1) by oxidation catalyzed by $CuSO_4$ is a consequence of the formation of an intramolecular disulfide bond in GLTP molecule.

Modification of GLTP with $S_4O_6^{2-}$ resulted in a partial inhibition (about 50%) of the transfer activity and a slight decrease in the binding affinity to PyrGalCer. This partial inactivation of GLTP is thought to be caused mainly by a decrease in the rate of formation and decomposition of the GLTP-PyrGalCer complex. The $Na_2S_4O_6$-treated GLTP fully restored both transfer and binding activity by reduction with 2-mercaptoethanol under non-denaturing conditions, which indicates that $Na_2S_4O_6$ produces a reversible protection of sulfhydryl group present on the surface of GLTP. The conversion of form E to form F resulted in the activation of the transfer activity and an increase of the binding affinity to PyrGalCer. Therefore, the activation of transfer activity seems to be a result of the enhancement of the formation of the GLTP-PyrGalCer complex and of increase in the turnover rate of the complex.

It is a problem to estimate the extent of activation of GLTP activity as a result of the formation of an intramolecular disulfide bond. GLTP treated with NEM consists of 70% of form C and 30% form D (Fig. 2, lane 2). After oxidation of NEM-treated GLTP of Cu^{2+}, form C was completely converted to form D and the transfer activity increased 2.4-fold when compared with that found before the oxidation. If the transfer activity of form C is assumed to 1, the following equations can be obtained

$$0.3a + 0.7 = b \quad (1)$$

$$a = 2.4b \quad (2)$$

where a is the transfer activity of form D, b is the transfer activity of GLTP treated with NEM. The transfer activity of form D is calculated to be 6. By the same method, we can also estimate the transfer activity of form E and form F. If the transfer activity of form E is assumed to 1, then, that of form F is calculated to be 4.2. Therefore, it might be assumed that the transfer activity of the faster component (form B) is 5-times as high as that of the slower component (form A).

In the preceding paper [11], we reported that the formation of GLTP oligomers by Cu^{2+} oxidation resulted in a remarkable decrease in the transfer activity and an increase in the affinity to PyrGalCer. It must be that both the formation of an intermolecular disulfide bond and the chemical reaction by NEM or tetrathionate involve the same sulfhydryl group in GLTP molecule. It is thought that oligomers have not only intermolecular but also intramolecular disulfide bond(s). These suggest that the sulfhydryl group reactive with thiol reagents in the GLTP molecule might affect the turnover rate of the GLTP-glycolipid complex, and two other sulfhydryl groups involved in the 2 SH ⇌ S-S

conversion might modulate the ability to bind glycolipid.

It was expected that the formation of an intramolecular disulfide bond might accompany a conformational change in GLTP molecule. Circular dichroism measurements indicated that GLTP had a high α-helix content and that the secondary structure of GLTP was different from that of phosphatidylcholine transfer protein from bovine liver, which contained 29% α-helix and 58% β-strand [10]. No significant change in ellipticity spectrum was brought about either upon the modification by $S_4O_6^{2-}$ or upon the formation of an intramolecular disulfide bond in GLTP: the change in α-helix and β-structure contents was small. This suggests that the formation of an intramolecular disulfide bond results in only a small change in the secondary or tertiary structure of GLTP.

The results presented in the preceding and present papers indicate the following: (1) GLTP consists of form A, major component and form B, minor component; (2) form A contains three sulfhydryl groups, one of which is sensitive to thiol reagents under non-denaturing conditions; (3) form B is formed from form A and contains one disulfide bond [11]. Conformational change of GLTP by the formation of an intramolecular disulfide bond is small. However, the formation of the disulfide bond significantly affects both the affinity of GLTP to glycolipids and the transfer activity. It is likely that the interaction of GLTP with membranes is also affected by the presence of disulfide bond.

In this paper, we used the faster component (form D or form F) modified with NEM or $S_4O_6^{2-}$ as a model of form B. It is assumed that form B with a free surface sulfhydryl group also behaves like the modified faster component. There is a possibility that the transfer activity of GLTP is regulated by the redox state of sulfhydryl groups in GLTP.

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