Biochemical Characterization of the Cys^{138}Arg Substitution Associated with the AB Variant Form of G_{M2} Gangliosidosis: Evidence That Cys^{138} Is Required for the Recognition of the G_{M2} Activator/G_{M2} Ganglioside Complex by β-Hexosaminidase A[†]

Bei Xie,[‡,§] Brigitte Rigat,[‡] Natasha Smiljanic-Georgijev,[‡,§] Huinan Deng,[‡] and Don Mahuran*[,‡,§]

Research Institute, Hospital for Sick Children, and Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Ontario, Canada

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ABSTRACT: The function of the G_{M2} activator protein is to act as a substrate-specific cofactor in the hydrolysis of G_{M2} ganglioside by β-hexosaminidase A. Mutations in the gene encoding it result in the AB variant form of G_{M2} gangliosidosis. One such mutation, Cys^{138}Arg, results in the mutant protein being retained and degraded in the endoplasmic reticulum of mammalian cells. In order to characterize the biochemical effects of this substitution, we expressed the mutant protein in transformed bacteria. We first compared the wild-type protein produced by two bacterial expression methods, one requiring protein refolding, with activator purified from the medium of transfected CHO cells. The "activity" and circular dichroism spectrum (α-helical content) of all three proteins were similar, justifying the use of refolded activator from transformed bacteria in structure/function studies. Second, the mutant protein was expressed in both bacterial systems and in each retained ~2% of the wild type's specific activity. The presence of even this small amount of activity in the mutant protein coupled with a calculated α-helical content nearly identical to the wild type, strongly suggest that no major tertiary or secondary structural changes, respectively, had occurred due to the mutation. However, we demonstrate that its heat stability at 60 °C is reduced 14-fold, suggesting some localized change in tertiary structure. The loss of a disulfide loop was confirmed by reacting the mutant protein with Ellman's reagent. A kinetic analysis detected a large increase in the apparent K_m of β-hexosaminidase A for the mutant; however, there was no apparent change in V_{max}. A fluorescence dequenching assay was used to evaluate the ability of the mutant protein to transport lipids and bind G_{M2} ganglioside. These assays detected no difference between the wild-type and mutant proteins, indicating that the Cys^{138}Arg substitution has no effect on these functions. We conclude that the mutation specifically affects a domain in the activator protein that is responsible for the recognition of the activator−G_{M2} ganglioside complex by β-hexosaminidase A.

The G_{M2} activator protein (activator)[1] is a substrate-specific cofactor for lysosomal β-hexosaminidase A (Hex A) in its hydrolysis of GalNAcβ(1−4)-[NeuAcα(2−3)-]-Galβ(1−4)-Glc-ceramide (G_{M2} ganglioside, G_{M2}) (Meier et al., 1991). It first solubilizes individual molecules of ganglioside or glycolipids by interacting with both their hydrophilic oligosaccharide and their hydrophobic ceramide moieties. Its specificity, *i.e.*, strength, of binding is primarily determined by the oligosaccharide moiety of the ligand, *e.g.*, $G_{M2} >> G_{M1} > G_{D1a} = G_{M3} = G_{A2}$ (Conzelmann et al., 1982; Conzelmann & Sandhoff, 1979). The activator can then replace its bound glycolipid in another membrane, thus acting as a general sphingolipid transport protein (Conzelmann et al., 1982; Smiljanic-Georgijev et al., 1997). When G_{M2} is the ligand the 1:1 activator−ganglioside complex can specifically interact with Hex A, resulting in the hydrolysis of G_{M2} to G_{M3} [reviewed in Sandhoff et al. 1995)]. Thus, its functional assay (the enhancement of G_{M2} hydrolysis by Hex A) is actually a measure of at least three separate activator binding functions, *i.e.*, oligosaccharide (for both NeuAc and GalNAc) binding, ceramide (lipid) binding, and Hex A binding. We have recently demonstrated that its lipid and oligosaccharide binding functions can be assessed independently using a fluorescence dequenching assay. It was shown that various glycolipids inhibit the ability of the activator to transport a self-quenching fluorescent lipid probe, octadecylrhodamine (R-18), between liposomes. Thus the rate of R-18 transport measures lipid binding, and the level by which various glycolipid inhibit this transport is a measure of oligosaccharide-binding (Smiljanic-Georgijev et al., 1997).

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* To whom correspondence should be addressed at Research Institute, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8. Telephone: 416-813-6161. FAX: 416-813-5086.

[‡] Research Institute, Hospital for Sick Children.

[§] University of Toronto.

[1] Abbreviations: activator, G_{M2} activator protein; Hex A, hexosaminidase A; GalNAcβ(1−4)-[NANAα(2−3)-]-Galβ(1−4)-Glc-ceramide; G_{M2} ganglioside; ER; endoplasmic reticulum, CD, circular dichroism; 5,5′-dithiobis(2-nitrobenzoic acid), Ellman's reagent or DTNB, MUG, 4-methylumbelliferyl β-*N*-acetylglucosamine; R-18, rhodamine conjugated to a saturated C-18 hydrocarbon chain; PC-LUV$_s$, phosphatidylcholine containing large unilamellar vesicles.

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The activator contains a signal peptide (residues 1−23) and is thus synthesized in the endoplasmic reticulum (ER) as a precursor polypeptide (residues 24−193). Its primary sequence contains a single site for N-linked glycosylation at Asn^{63}. The presence of the oligosaccharide increase the M_r of the precursor, as determined by SDS−PAGE, to 24 000. Amino-terminal processing in the lysosome to the mature form reduces its M_r to 22 000 (residues 32−193) (Burg et al., 1985; Xie et al., 1991). The critical role in human metabolism played by the activator is demonstrated by the occurrence of a fatal autosomal recessive disease, the AB variant form of G_{M2} gangliosidosis, that results from mutations in the *GM2A* gene encoding the activator (Schepers et al., 1996; Schröder et al., 1993, 1991; Xie et al., 1992). A $Cys^{138}Arg$ substitution was the first mutation to be described in cells from an acute G_{M2} gangliosidosis, AB variant patient (Schröder et al., 1991; Xie et al., 1992). Expression studies of the mutant protein in COS cells demonstrated that the substitution prevented intracellular transport of the precursor out of the ER and greatly accelerated its degradation (Xie et al., 1992).

The retention through recycling between the ER and the cis-Golgi network of unassembled and/or misfolded subunit-(s) is thought to be achieved through interactions with resident proteins, chaperones, which are themselves normally recycled between these compartments (Lewis & Pelham, 1990; Munro & Pelham, 1987). The presence of this "quality control system" suggests that the $Cys^{138}Arg$ substituted activator may not of necessity be incapable of forming a partially functional protein but may be prevented from doing so by its increased affinity for one or more chaperone proteins. This is apparently the case for the plasma membrane protein CFTR carrying the ΔPhe^{508} mutation (the major mutation causing cystic fibrosis) (Ward et al., 1995), which if expressed at lower temperatures or in nonmammalian cells reaches the plasma membrane and is partially functional (Cheng et al., 1990). This same mechanism has been shown to be the cause of other forms of G_{M2} gangliosidosis resulting from mutations in either of the two genes encoding the α (Tay-Sachs, *HEXA*) or β (Sandhoff, *HEXB*) subunits of Hex A [reviewed in Gravel et al. (1995) and Mahuran (1991)].

The wild-type activator has been produced in transformed bacteria and methods for refolding of a functional protein developed (Klima et al., 1993; Smiljanic-Georgijev et al., 1997; Wu et al., 1994b, 1996). In this report we demonstrate the validity of using refolded activator protein from transformed bacteria to investigate the biochemical effects of a naturally occurring mutation in the activator, $Cys^{138}Arg$. Our data suggest that the presence of the quality control system in the ER may increase the clinical severity associated with this mutation and identify a critical role for Cys^{138} or the disulfide loop it forms in the interaction between the activator−G_{M2} complex and Hex A.

MATERIALS AND METHODS

Activator Proteins. The G_{M2} activator protein purified from transfected CHO cell medium (CHO-activator) and from an *Escherichia coli* expression system that allowed synthesis, purification, and refolding of a His_6−mature (S^{32}−I^{193}) G_{M2} activator fusion protein (His_6−activator, *i.e.*, $MRGS(H)_6GSIEGR\text{-}S^{32}$−$I^{193}$) were obtained as previously reported (Smiljanic-Georgijev et al., 1997). A nonfunctional, truncated form of the activator also produced using the His_6 system, was used as a negative control. This form resulted from a Taq error encoding a frameshift mutation after Leu^{157}, which then also allows 13 new residues to be translated before a stop codon is encountered, *i.e.*, $MRGS(H)_6GSIEGR\text{-}S^{32}$−$L^{157}$-WSCPVGSPPGTTA (Smiljanic-Georgijev et al., 1997). Secondary structure predictions were carried out with programs contained in GeneWorks (IntelliGenetics).

Expression of the Functional FLAG−Activator Fusion Protein in E. coli. Oligos 217, 5′-GGCTCGAGTGGGAGTTTGGCCTTGGCAA, and 218, 5′-GGAAGCTTCACCTGAAAAAGCCATCCCA, were used to amplify the coding sequence (residues His^{24}−Ile^{193}) of the activator precursor contained in the plasmid pAct1 (Xie et al., 1991), by PCR. For subcloning, a *Xho*I or a *Hin*dIII site (underlined) was introduced into each of the PCR primers. The PCR fragment was subcloned in the *E. coli* expression vector pFLAG-1 (IBI, catalog no. IB 13003), in frame with an amino-terminal extension that encodes the 21 amino acid OmpA signal peptide (for export to the periplasmic space) and the FLAG peptide (D-Y-K-D-D-D-D-K). Additionally two other residues, Lys-Leu, are encoded by the *Hin*dIII site before the beginning of the activator sequence. The insert from one clone was confirmed by nucleotide sequencing to contain no Taq errors. Expression and purification of the fusion protein from this clone were carried out according to the protocol provided by the company (IBI, catalog no. IB 13000). Basically, about 50 mL of overnight culture was added to 500 mL of LB medium and grown until log phase (OD_{260} = 0.6−0.8). IPTG was added to 2 mM and the bacteria cells were harvested when the OD_{260} reached 1.0. The fusion protein was extracted from the periplasmic space and cell lysate, respectively, and then purified by passage through a FLAG monoclonal antibody column, according to the protocol. The eluted fractions were collected and concentrated by Centricon 10 microconcentrator (Amicon, product no. 4206).

Expression of the FLAG− and His_6−$Cys^{138}Arg$ Mutant Activator Fusion Proteins in E. coli. A soluble form of the AB variant, $Cys^{138}Arg$ mutant activator protein was obtained from the periplasmic space of transformed bacteria and purified using the FLAG system. Oligos 217 and 218 were used to amplify by PCR the coding sequence of the mutant activator precursor contained in $pAct_{M1}$, the plasmid that was previously used for expression of the mutant activator in COS cells (Xie et al., 1992). The PCR fragment was then subcloned into pFLAG-1. Additionally, an insoluble form of the mutant activator was purified and refolded from bacteria transformed with the His_6 fusion vector, pQE-8 (Qiagen). The coding sequence in $pAct_{M1}$ was amplified using primers that allowed it to be inserted in-frame and downstream from the nucleotides encoding the His_6 sequence, as previously reported for the wild-type activator (Smiljanic-Georgijev et al., 1997). Prior to subcloning, the two PCR fragments were confirmed by nucleotide sequencing to contained the $T^{412}C$ transition with no further changes due to Taq error.

Circular Dichroism Spectra. CD spectra were recorded on a Jasco J-720 spectropolarimerer, with a protein concentration of 0.1−0.3 mg/mL in 80 mM citrate phosphate (pH

4.1) buffer. Each curve was the average of four scans, recorded between 190 and 250 nm in a quartz cell (Jasco) with a path length of 1 mm. The content of each secondary structure was obtained from a program in the instrument that used Yang's method of calculation (Yang et al., 1986). However, only the calculations for α-helix content are reported. This is because with data extending to only 190 nm, the α-helix content is the only secondary structure that can be determined with confidence (Johnson, 1990).

Heat Stability Based on the Ability of the Activator To Serve as a Cofactor for Hex A. The purified normal or AB variant mutant activator−FLAG fusion protein was added to 250 μL of 80 mM citrate phosphate buffer, pH 4.1, containing 0.1% human serum albumin to generate final concentrations of 1 and 10 ng/μL, respectively. Aliquots (50 μL) were removed at various intervals after incubation at 60 °C (0, 15, 30, and 60 min for normal activator and 0, 4, 8, and 13 min for the mutant activator), placed on ice, and used for the functional assay of the activator in the presence of ^{3}H-G_{M2} and purified placental Hex A, as previously described (Smiljanic-Georgijev et al., 1997). For the calculation of each protein's half-life ($T_{1/2}$), incubation time was plotted versus the log of the percent G_{M2} hydrolysis remaining. The best-fit line was generated by least-squares analysis and the time at which 50% of activator function remained ($T_{1/2}$) was calculated.

Determination of the Number of Free Sulfhydryl Groups in the Activator. DTNB (4 μg) was used to detect free sulfhydryl groups present after the refolding of the wild-type and Cys^{138}Arg-substituted His$_6$−activators. The method used was based on Ellman's original procedure (Ellman, 1959) with the following exceptions: (a) each protein was incubated (overnight at 4 °C) with 2 mM reduced glutathione, precipitated with 4 volumes of acetone, washed 5× with 80% acetone, dissolved in 2% SDS in phosphate buffer, pH 8.0, and heated for 2 min at 100 °C; (b) three samples (7, 14, and 27 μg) of each protein were analyzed and the best-fit line was calculated to obtain the number of free Cys (based on an extinction coefficient at OD_{412} of 13 600 M^{-1}); and (c) the total reaction volume was 100 μL.

Western Blot Analysis. Samples of the lysate or the periplasmic space of transformed bacteria were mixed with sample buffer containing 3% SDS and 25 mM DTT and boiled for 5 min. The proteins in each sample were separated by SDS−PAGE using the Laemmli gel system (10% gel) (Laemmli, 1970). The proteins were transferred to nitrocellulose overnight (Brown et al., 1989). Western blotting was carried out as previously described using the Amersham ECL system (Xie et al., 1992).

Kinetics of Hex A Hydrolysis of G_{M2} from the Ganglioside−Activator Complex. The wild-type (0−2000 ng, 0−0.11 nmol) and the Cys^{138}Arg (0−5000 ng, 0−0.27 nmol) substituted activator proteins were assayed for functionality using purified placental Hex A (Mahuran & Lowden, 1980) [50 000 nmol of 4-methylumbelliferyl β-*N*-acetylglucosamine (MUG)/h] and ^{3}H-G_{M2} ganglioside (20 nmol) in a total reaction volume of 100 μL (Hou et al., 1996). Kinetic constants were calculated using a computerized nonlinear least-squares curve-fitting program for the Macintosh, KaleidaGraph 3.08c. Thus the individual activator concentrations and their corresponding initial velocity measurements were directly fitted to the Michaelis−Menten equation, $V_i = V_{max}[\text{activator}]/(K_m + [\text{activator}])$, making possible the calculation of an accurate standard error for the apparent K_m and V_{max} values (Hou et al., 1996; Tommasini et al., 1985).

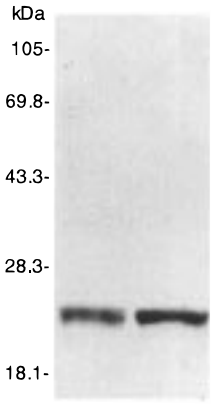

FIGURE 1: Purified wild-type (left lane) and AB variant, Cys^{138}-Arg, mutant activator (right lane) produced in transformed bacteria by the FLAG system, analyzed by SDS−PAGE and visualized by Coomassie blue staining.

Ganglioside Binding. The ability of 4 μg (0.21 nmol) of the wild-type and mutant activator protein to bind 2 μg (1.3 nmol) of G_{M2} ganglioside was assessed at pH 5 using a fluorescence dequenching assay. This assay first evaluates the ability of the activator to transport a self-quenching fluorescence dye, rhodamine conjugated to a saturated C-18 hydrocarbon chain (R-18), between labeled and unlabeled liposomes (phosphatidylcholine containing large unilamellar vesicles, PC-LUV$_s$). If the transporter function is present, *i.e.*, the hydrophobic binding site is functional, the protein's ability to bind G_{M2} is assessed by using the ganglioside as a specific inhibitor of the process; *i.e.*, the assay evaluates the protein's oligosaccharide as well as its hydrophobic binding site (Smiljanic-Georgijev et al., 1997). The fluorescence intensity, 5 min after addition of R-18 PC-LUV$_s$ to the reaction mix containing the activator (± G_{M2}) and unlabeled PC-LUV$_s$, is taken as F_0. At the end of the reaction Triton X-100 is added to the assay mixture to obtain the value at infinite dilution of the probe (F_{100}). Fluorescence measurements are taken at various time points (F_t) and the dequenching for each point calculated as % fluorescence (R-18) dequenching = 100 $(F_t - F_0)/F_{100}$ (Smiljanic-Georgijev et al., 1997).

RESULTS

We have previously compared the ability of the purified activator isolated from the medium of transfected CHO cells and the refolded bacterial His$_6$−activator to enhance the hydrolysis of G_{M2} by Hex A, *i.e.*, compared the specific "activity" of these forms of the activator. We found the His$_6$−activator to have 52% the specific activity of the CHO-activator (Smiljanic-Georgijev et al., 1997). We next evaluated the FLAG bacterial expression system. Unlike the His$_6$−activator, the FLAG−activator fusion protein (FLAG−activator) remained soluble and was purified from both the lysate and the periplasmic space of the transformed bacteria (Figure 1). The activity of the FLAG−activator from both locations in the bacteria was compared. Whereas the activator from the periplasmic space was functional, a similar amount (as judged by Western blotting, bottom of Figure 2) of the fusion protein from the bacterial lysate showed little

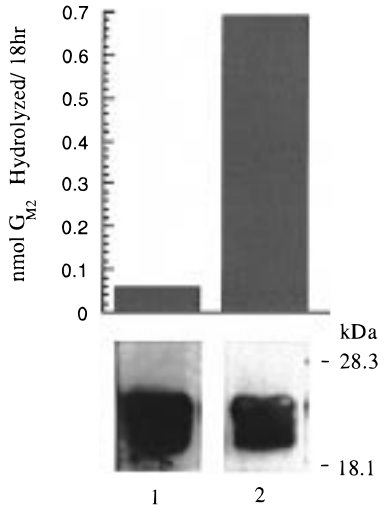

FIGURE 2: Effects of small but similar amounts of the FLAG−activator fusion protein (as judged by Western blot analysis shown in the lower panel) contained in either the bacterial lysate (lane 1) or the periplasmic space (lane 2), on the ability of purified Hex A to hydrolyze ^{3}H-G_{M2} ganglioside (upper panel).

activity (top of Figure 2). The specific activity of the purified activator from the periplasmic space was found to be 67% of the CHO cell-produced activator or 130% of the refolded His_6−activator (Table 1). However, the overall yield was low, <200 μg/L of culture. A comparison of the CD spectra of activator produced by all three of the above systems demonstrated that each method produced activator proteins with nearly identical α-helical contents (Figure 3, Table 1). Taken together, these data indicate that neither bacterial expression nor refolding of the activator appears to have a major effect on either its secondary (CD) or tertiary (activity) structure.

Since the FLAG system produced a functional activator without the need for refolding, we initially expressed the AB variant, Cys^{138}Arg form of the activator using this method. The system produced soluble mutant activator that could be purified from the periplasmic space on the FLAG monoclonal antibody column (Figure 1). The specific activity of the AB variant activator was determined to be 1.3% of the wild-type FLAG-activator (Table 1). A comparison with similar large amounts of the inactive truncated form of the activator [made in the His_6 system (Smiljanic-Georgijev et al., 1997)] confirmed the significance of this low level of activity (Figure 4, Table 1). Since activity could

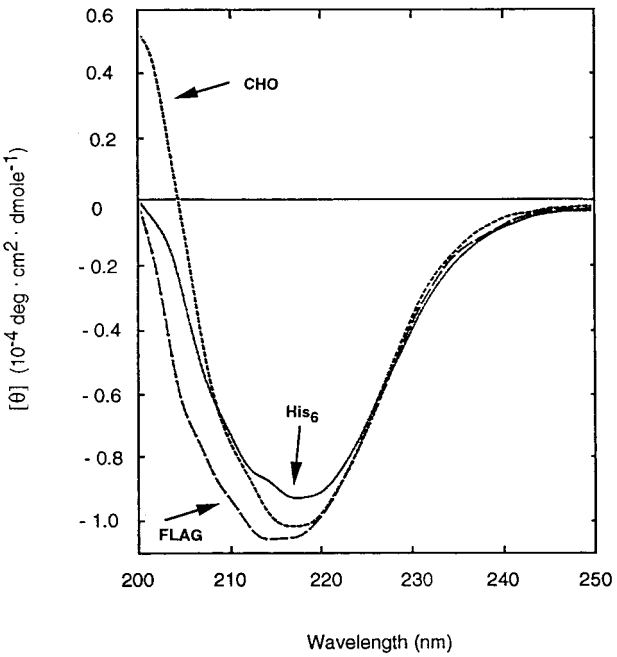

FIGURE 3: CD spectra of the FLAG− and His_6−activator fusion proteins from transformed bacteria and of activator purified from the medium of transfected CHO cells (see Table 1).

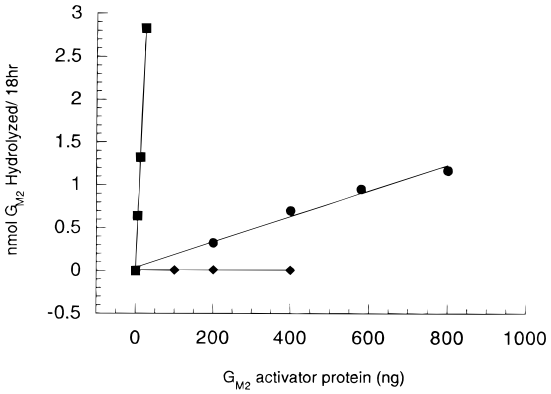

FIGURE 4: Hydrolysis of G_{M2} ganglioside over 18 h by purified Hex A (10^5 units) in the presence of various amounts of wild-type (■), Cys^{138}Arg (●), or the truncated (◆) forms of the activator protein. The actual data points are shown with the best-fit straight line (least squares) drawn between them. The slopes ± standard error were used to calculate specific activity (Table 1).

be measured in the Cys^{138}Arg mutant, as well as the wild-type activator, we next determined their heat stability at 60

Table 1: Summary of the Biochemical Characteristics of Various Forms of the Activator Protein

	CHO-	FLAG-	His_{6-}	FLAG−AB	His_6−AB	His_6-trunc
cofactor for Hex A[a]	9.2 ± 0.6[b]	6.2 ± 0.2	4.8 ± 0.3	0.11 ± 0.01	0.083 ± 0.006	0.0004 ± 0.0007
% α-helical (CD)	32.5	25.9	25.6	25.1	ND[c]	13.7
$T_{1/2}$ 60 °C	ND	43	ND	3.0	ND	
mol of SH/mol of activator[d]	ND	ND	0.4	ND	1.3	ND
K_m[e] (nM activator)	ND	ND	170 ± 20	ND	2000 ± 500	
V_{max}[e] (nmol of G_{M2}/h)	ND	ND	0.30 ± 0.01	ND	0.37 ± 0.04	
% R-18 transport[f]	ND	ND	100	ND	95 ± 5	4[g]
% inhibition (G_{M2})[h]	ND	ND	83 ± 1	ND	80 ± 3	0

[a] Nanomoles of G_{M2} hydrolyzed by 10^5 units of Hex A h^{-1} μg^{-1} (activator). Measures a combination of three binding binding functions (see footnotes *e*, *f*, and *h*). [b] ± Standard error. [c] ND, not determined. [d] After the reaction of the activator with DTNB. [e] Apparent K_m (ability of Hex A to bind the activator−ganglioside complex) and V_{max} (maximum rate of G_{M2} hydrolysis by Hex A, 5×10^4 units). [f] Percent of the normal activator's (4 μg) rate of transport of R-18 between liposomes; measures the lipid transport function of the activator. [g] Twelve-fold higher levels of the truncated activator (His_6-trunc) only doubled this rate (Smiljanic-Georgijev et al., 1997). [h] Percent inhibition of R-18 transport between liposomes when 1.3 nmol of G_{M2} is preincubated with the activator; measures the oligosaccharide binding function of the activator.

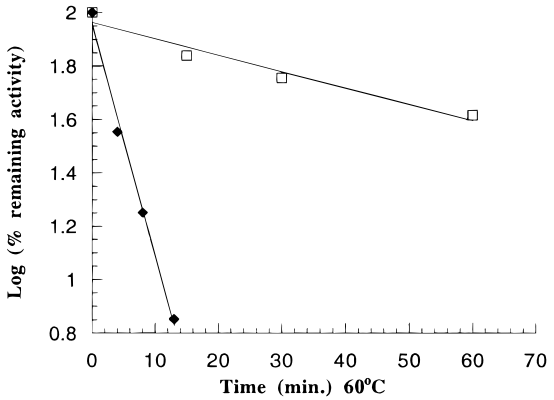

FIGURE 5: Heat stability as measured by changes in the enhancement of Hex A activity toward G_{M2} ganglioside [plotted as log (% remaining activity); the activity at 0 time was taken as 100%], of the wild-type (1 ng/μL, □) and the Cys^{138}Arg mutant (10 ng/μL, ◆) FLAG−activator proteins at 60 °C. $T_{1/2}$ values (Table 1) were calculated from each best-fit line (least squares).

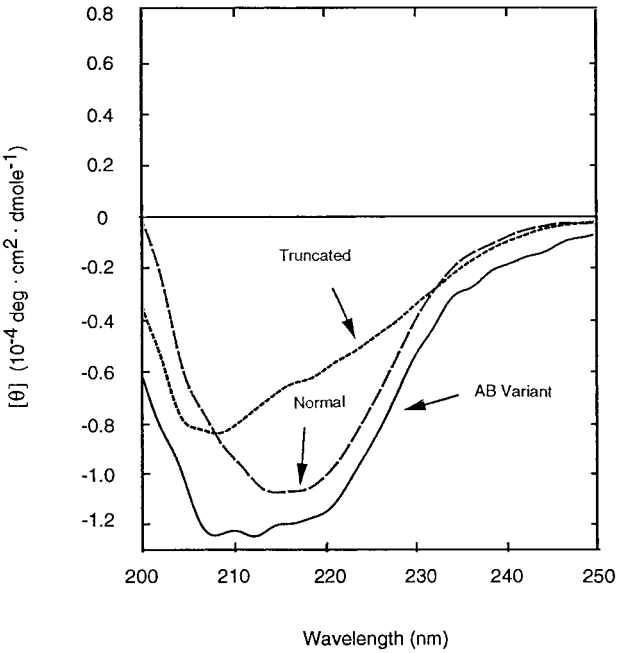

FIGURE 6: CD spectra of the wild-type and Cys^{138}Arg mutant FLAG−activator proteins compared with the nonfunctional His_6-truncated form (see Table 1).

°C [using 10-fold more of the mutant (Figure 5)]. The $T_{1/2}$ for the wild-type protein was 14-fold longer than that for the Cys^{138}Arg mutant activator (Table 1). Each form was found to be stable for at least 1 h at 37 °C (data not shown). Finally the CD spectrum of the mutant protein was analyzed and compared to the wild-type and to the nonfunctional (Figure 4) His_6-truncated form of the protein (Figure 6). The α-helical content for the AB variant activator and the truncated form were calculated to be 97% and 53%, respectively, of that calculated for the wild-type protein (Table 1). Thus the mutation does not have a major (global) effect on secondary structure. Furthermore, the presence of even this small amount of activity suggests that the mutation also does not have a major effect on the tertiary structure of the activator; however, the loss of heat stability does suggest a small localized change, *e.g.*, the loss of a disulfide loop. The loss of 50% of the wild type's helical structure in the truncated form indicates either that there is a global disruption in its folding patterns or that the deleted C-terminal section is normally an α-helix in the wild-type activator. The loss of all assayable functions makes it impossible to differentiate between these possibilities.

Because of the low yield with the FLAG system, the Cys^{138}Arg-substituted activator was expressed in the His_6 system and refolded after purification on the Ni^{2+} column. The yield of mutant activator from this procedure was similar to what we had previously obtained for the wild-type protein, ∼50 mg/L (Smiljanic-Georgijev et al., 1997). The activity of the refolded, mutant His_6−activator was similar to that of the mutant FLAG−activator (Table 1). Thus, refolding of the Cys^{138}Arg mutant activator does not appear to either enhance or decrease the effects of the mutation on the activator.

In order to confirm that the Cys^{138}Arg substitution interrupts a disulfide bond, the refolded normal and mutant forms of the His_6−activator were denatured in SDS and reacted with DTNB for 15 min. The wild-type activator produced a small increase in OD_{412} over the incubation period equivelent to 0.4 mol of free sulfhydryl groups/mol of protein, indicating that all of its eight Cys groups normally form disulfides. On the other hand the reaction of DTNB with the mutant activator produced an increase in OD_{412} equivelent to 1.3 mol of free sulfhydryl groups/mol of protein, indicating the presence of a single free Cys (Table 1).

The wild-type and mutant His_6−activators were next assayed over a wide range of concentrations with constant amounts of Hex A and ^{3}H-G_{M2} (Figure 7), and the data were fitted to the equation G_{M2} hydrolyzed/h = V_{max}[activator]/(K_m + [activator]). Surprisingly, the calculated V_{max} values were nearly identical using either the wild-type (Figure 7A) or mutant (Figure 7B) proteins as cofactors for Hex A (Table 1). The difference in activity between the two forms appeared to be totally caused by a change in the affinity of Hex A for the mutant activator (Figure 7, Table 1). Since the apparent K_ms actually represent a combination of two binding constants, *i.e.*, the activator toward G_{M2} and Hex A toward the activator−G_{M2} complex, a further experiment was performed to identify which had been affected by the mutation. We used our previously reported fluorescence dequenching assay to determine the ability of each protein to transport R-18 between liposomes and then tested the levels of transport inhibition caused by the addition of G_{M2} (Smiljanic-Georgijev et al., 1997). Virtually identical rates of R-18 transport and G_{M2} inhibition were observed (Figure 8, Table 1), indicating that the mutation does not affect the activator's general lipid transport function or its oligosaccharide specificity, *i.e.*, ability to bind G_{M2} ganglioside.

DISCUSSION

Several laboratories have reported the production of activator from transformed bacteria, but each procedure included a refolding step in order to obtain a functional protein (Klima et al., 1993; Smiljanic-Georgijev et al., 1997; Wu et al., 1994b, 1996). In this report we demonstrate that if the activator is exported to the periplasmic space of the transformed bacteria, a functional protein can be obtained without refolding (Figure 2). Unfortunately the yield using this methodology (FLAG) was 250-fold lower than was

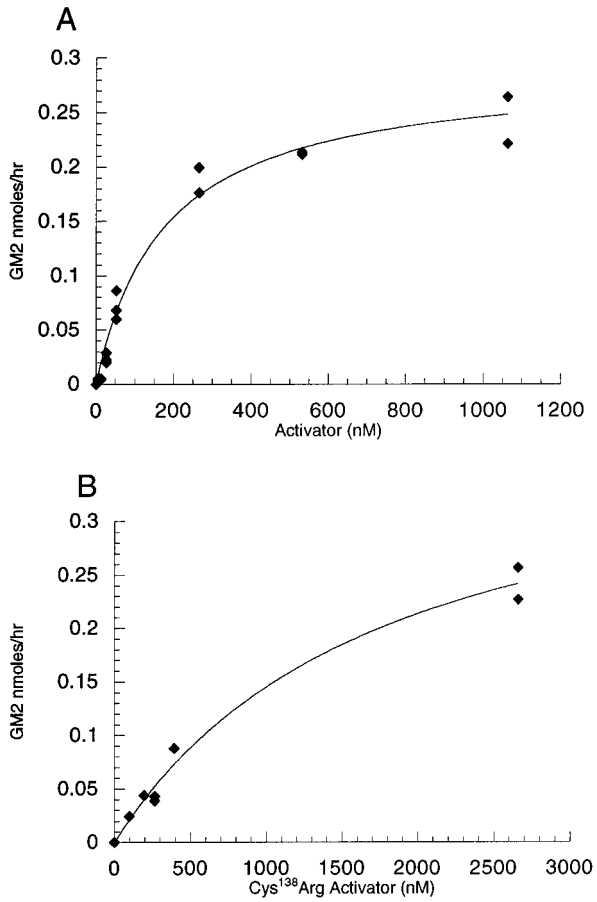

FIGURE 7: Kinetic analyses of (A) the wild-type and (B) the Cys^{138}-Arg mutant activator proteins. Varying amounts of activator (*x*-axis) were used to enhance the hydrolysis of a constant amount of G_{M2} ganglioside by a constant amount of Hex A (5×10^4 units). The actual data points are shown with the best-fit curve drawn between them. The data weremodel-fitted and the apparent V_{max} and K_m values (± standard error) were calculated (Table 1).

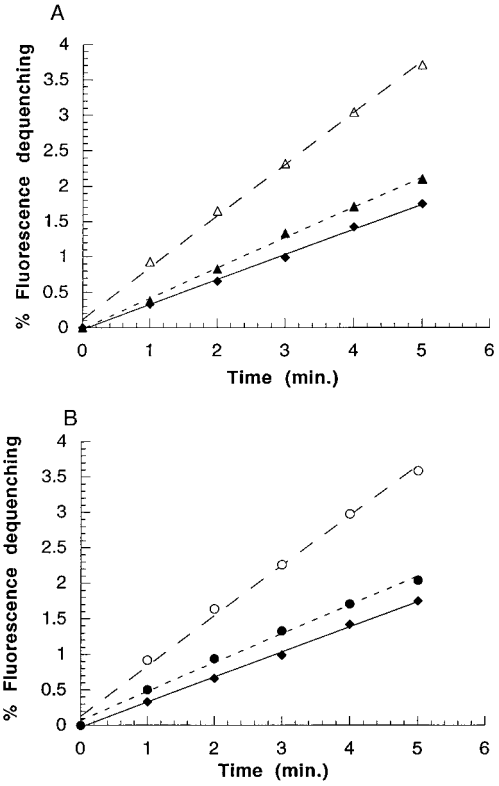

FIGURE 8: Percent fluorescence (R-18) dequenching of (A) the wild-type (triangles) and (B) the Cys^{138}Arg mutant (circles) activator proteins in the presence (solid symbols) or absence (open symbols) of added G_{M2} ganglioside. Baseline dequenching is shown in each panel as (◆). The actual data points are shown for each with the best-fit straight line (by least squares) drawn between them. The calculated slopes (± standard error) were 0.76 ± 0.02 and 0.42 ± 0.005 (in the presence of G_{M2}) for the wild type and 0.74 ± 0.02 and 0.43 ± 0.01 (in the presence of G_{M2}) for the mutant. The baseline slope (no activator present) in both cases was 0.35 ± 0.005 (see Table 1).

obtained using the His_6 system. Whereas the cytosol of both bacterial and mammalian cells is a reducing environment, there is an oxidizing environment in the bacterial periplasmic space of and the mammalian ER. Thus, disulfide bond formation is rare in proteins synthesized in either cells' cytosol. This is likely a major reason for the need to refold. Consistent with this idea was the low level of detectable free sulfhydryl groups in the pool of refolded His_6−activator (Table 1) and our ability to refold to a functional protein, the FLAG−activator isolated from the bacterial cytosol. However, yields were still 50-fold lower than from the His_6 system (data not shown). Furthermore, the capacity of the monoclonal anti-FLAG column was low and its price high, making the system generally unattractive for preparative work.

Despite the FLAG system's drawbacks, it was of interest to compare the FLAG−activator protein from the periplasmic space (nonglycosylated but no refolding needed) with activator we had previously isolated from the medium of transfected mammalian (CHO) cells (glycosylated) or isolated and refolded from transformed bacteria using the His_6 system (Smiljanic-Georgijev et al., 1997). Measurements of the ability of each activator to serve as a substrate-specific cofactor for Hex A, reflective of their overall tertiary structures, and their CD spectra, reflective of secondary structure, were compared and found to be similar (Table 1). We conclude that neither their lack of a carbohydrate nor their different N-terminal extensions or requirement for refolding of the protein was a significant factor in producing an activator in transformed bacteria nearly identical to the mammalian protein. This conclusion has been strengthened by two recent articles that demonstrate that a significant amount of newly synthesized activator is secreted by mammalian cells. Furthermore, this pool contains activator molecules with varying types of, or even no, oligosaccharide (Glombitza et al., 1997; Rigat et al., 1997). Thus the single oligosaccharide is not required for its function or for its intracellular transport and secretion from mammalian cells.

Missense mutations that occur in proteins synthesized in the ER of mammalian cells often result in the proteins' retention and rapid degradation [reviewed in Lodish (1988) and Mahuran (1991)]. Since we have shown that this is also true for the Cys^{138}Arg substitution mutation associated with the AB variant form of acute G_{M2} gangliosidosis (Xie et al., 1992), it was not possible to obtain sufficient activator from transfected mammalian cells to fully characterize the direct biochemical effects of the mutation on activator function. Expression of the mutant activator in transformed bacteria appeared to be the solution to this problem. However, we first determined if refolding was as valid a procedure to use with a mutant form of the protein as it was with the wild-type activator (discussed above). This appears to be the case,

as the $Cys^{138}Arg$ activator produced by the His_6 system (refolded) had the same low specific activity found for the protein produced by the FLAG system (no refolding) (Table 1).

The locations of the domains in the activator responsible for its three binding functions, *i.e.*, lipid, oligosaccharide, and Hex A, have been suggested in a previous study by Wu et al. (1996). The NeuAc binding site was located within residues 34−142 and either the GalNAc or Hex A recognition site in the remaining residues 143−193. They also demonstrated that the activator does not recognize the oligosaccharide moiety from G_{M2} without the ceramide being present. Thus, it was suggested that the hydrophobic (lipid) binding pocket is also contained within residues 34−142 (Wu et al., 1996). This model would predict that the $Cys^{138}Arg$ substitution would either have a broad general effect on the overall folding of the protein or specifically affect ganglioside binding.

Our CD data plus the findings that mutant protein retains some activity (∼2% that of wild type, Table 1) indicate that there are no gross changes in the tertiary or secondary structure of the $Cys^{138}Arg$ mutant activator. However, the lower heat stability of the mutant and its increased reactivity to DTNB (Table 1) indicate that Cys^{138} is involved in a disulfide bond, and it would follow that its substitution by Arg would cause some, apparently localized, change in the tertiary structure of the protein. We next investigated the specific biochemical effect(s) of the substitution on each of the three activator binding functions. A kinetic examination of the mutant activator's ability to serve as a substrate-specific cofactor for Hex A demonstrated that an increase in the apparent K_m of Hex A for the mutant was fully responsible for its decreased specific activity (Figure 7, Table 1). These data suggested that the mutation was affecting either the ability of the activator to bind G_{M2} and form the complex, *i.e.*, the substrate for Hex A, or the interaction between Hex A and the G_{M2}−activator complex. To differentiate between these possibilities we used our previously developed fluorescent dequenching assay; the truncated form of the activator is nonfunctional in this assay (Table 1) (Smiljanic-Georgijev et al., 1997). The assay, $\pm G_{M2}$, produced identical results for the $Cys^{138}Arg$ mutant (Figure 8B) and wild-type (Figure 8A) activators (Table 1). Thus, Cys^{138} and the disulfide loop it forms are critically important for Hex A−activator interaction but not for G_{M2} ganglioside−activator interaction. These data further support our conclusion that the mutation causes only a small localized change in the activator's tertiary structure.

In another study, Fürst et al. (1990) suggested that one or more amphiphilic α-helices, predicted by computer analysis to be present in the activator, may form a hydrophobic binding pocket for the ceramide moiety. By use of the Garnier method (Garnier et al., 1978) to predict α-helix structures in the activator, two were identified between residues 80−95 and 108−128. By use of the DeLisi method for predicting amphipathic α helices (Cornette et al., 1987), only residues 108−128 qualify. However, an additional amphipathic α-helix was predicted between residues 168 and 178. Since in our truncated form of the activator a reading frameshift occurs after residue 157 and the helical content of the protein dropped to 50% that of the wild-type protein, it is likely that residues 168−178 do indeed form an amphipathic α-helix. The lack of ganglioside binding by the truncated activator (Smiljanic-Georgijev et al., 1997) would then suggest that this amphipathic α-helix is part of the activator's hydrophobic binding pocket. However, it is also possible that a more global change in secondary and tertiary structure has occurred.

The above conclusions do not support the model suggested by Wu et al. (1996), which places the Hex A recognition site at the C-terminus and the ganglioside binding domain near the N-terminus of the protein. However, our data, demonstrating that Cys^{138} plays a major role in the recognition of the complex by Hex A and linking residues 168−178 with ganglioside binding, are not irreconcilable with those of Wu et al. (1996). Their previous model considered the occurrence of only linear-type domains, *i.e.*, domains consisting of sequential residues within the protein's primary structure. It is also possible that protein folding and disulfide bond formations (our DTNB results demonstrate that all eight Cys residues in the activator are formed into disulfides; Table 1) could bring nonlinear sections of the protein together to form these binding domains. For example, the second amphipathic α-helix, residues 108−128, could form part of the hydrophobic binding pocket and is located in the region Wu et al. (1996) linked with ganglioside binding. As well, Cys^{138} could form a disulfide bond with Cys^{183} to produce the domain recognized by Hex A.

Our data demonstrate that even a small change in structure due to a point mutation, *i.e.*, not sufficient to eliminate overall activity, have any effect on ganglioside binding, or produce a significant change in the CD spectra, is still sufficient for a protein to be recognized as abnormal by the quality control system in the ER of mammalian cells. In the case of the $Cys^{138}Arg$ substitution in the activator, the mutant protein retains only ∼2% of the wild type's specific activity. However, similar small amounts of residual Hex A activity have been shown to produce a milder clinical phenotype, *e.g.*, subacute rather than acute, in patients with Tay-Sachs or Sandhoff disease [reviewed in Gravel et al. (1995)]. Thus the mechanism of retention and accelerated degradation of "abnormal" proteins in the ER is a factor exacerbating the course and severity of this as well as other genetic diseases, *e.g.*, some forms of Tay-Sachs (Brown & Mahuran, 1993; De Gasperi et al., 1996), mucopolysaccharidosis type VII (Wu et al., 1994a), and cystic fibrosis (Cheng et al., 1990).

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