Effect of Saposins A and C on the Enzymatic Hydrolysis of Liposomal Glucosylceramide

(Received for publication, February 25, 1997, and in revised form, April 28, 1997)

Anna Maria Vaccaro‡, Massimo Tatti, Fiorella Ciaffoni, Rosa Salvioli, Alessandra Barca, and Chiara Scerch

From the Department of Metabolism and Pathological Biochemistry, Istituto Superiore Sanita’, Viale Regina Elena 299, 00161 Roma, Italy

The degradation of glucosylceramide in lysosomes is accomplished by glucosylceramidase with the assistance of, at least, another protein, saposin C (Sap C), which is generated from a large precursor together with three other similar proteins, saposins A, B, and D. In the present study, we have examined the effects of saposins on the enzymatic hydrolysis of glucosylceramide inserted in large and small phospholipid liposomes. The glucosylceramide contained in large unilamellar vesicles (LUV) was degraded by glucosylceramidase at a rate 7–8-fold lower than glucosylceramide inserted in small unilamellar vesicles (SUV). The separate addition of either Sap A or Sap C to the LUV system partially stimulated the sphingolipid degradation while saposins B and D had no effect. In the presence of both Sap A and Sap C, the rate of sphingolipid degradation was higher than the sum of the rates with the two saposins individually, indicating synergism in their actions. The stimulatory effect of the two saposins depended on the incorporation of an acidic phospholipid such as phosphatidylserine (PS) into LUV.

The characteristics of glucosylceramidase activation by Sap C were different from those of Sap A. Sap C increased the rate of hydrolysis of both the artificial water soluble substrate, 4-methylumbelliferyl-β-D-glucopyranoside, and the lipid substrate, glucosylceramide, while Sap A only stimulated degradation of the sphingolipid. Also the binding properties of Saps A and C were markedly different. At acidic pH values, Sap C bound to PS-containing LUV and promoted the association of glucosylceramidase with the membrane. In contrast, Sap A had poor affinity for the membrane even in the presence of glucosylceramide; moreover, Sap A did not potentiate the capacity of Sap C to mediate glucosylceramidase binding.

In conclusion, our results show that both Sap A and Sap C are required for maximal hydrolysis of glucosylceramide inserted in PS-containing LUV, that their effects are synergistic, and that their mode of action is different. Sap C is responsible for the membrane binding of glucosylceramidase, while Sap A stimulation is possibly related to its effect on the conformation of the enzyme. It can be envisaged that Sap A in conjunction with Sap C might have a physiological role in glucosylceramide degradation.

Glucosylceramide is degraded in lysosomes by the concerted action of glucosylceramidase and, at least, another protein called saposin (Sap)1 C (1–5). A defective glucosylceramidase activity leads to glucosylceramide accumulation and Gaucher disease (1–5). Glucosylceramidase and Sap C are highly hydrophobic proteins that become water soluble after purification; immunocytochemical studies demonstrated that a large proportion of both proteins are associated with the lysosomal membrane (6, 7).

Sap C is released together with three other similar proteins, Saps A, B, and D, from a common precursor called prosaposin (1–4, 8–10). All saposins appear to be involved in the catabolism of sphingolipids (1–4). A patient lacking the four saposins in consequence of a mutation in the prosaposin initiation codon showed a combined sphingolipid storage disorder (11, 12). A selective deficiency of Sap C caused glucosylceramidase accumulation in a variant form of Gaucher disease (13). Mutations affecting the coding region of Sap B caused a variant form of metachromatic leukodystrophy with storage of sulfatides (14, 15). An isolated deficiency of either Sap A or D has not been reported so far, and thus the physiological consequences of the absence of one of these two saposins are unknown.

To explore the role of Sap A, the effect of this saposin on the degradation of sphingolipids has been tested in vitro. It was reported that Sap A is able to increase substrate hydrolytic rates of glucosylceramidase and galactosylceramidase (16), an observation that prompted the authors to propose an involvement of Sap A in the metabolism of gluco- and galactosylceramides (16). Another group claimed that Sap A was able to stimulate the glucosylceramidase activity at high concentrations (17) while at low physiological concentrations, it bound to glucosylceramidase without activating effects (18). It was thus considered unlikely that Sap A is an important activator of the enzyme.

While the function of Sap A is still debated, that of Sap C as glucosylceramidase activator is well assessed (1–5, 13). In vitro, the Sap C-induced stimulation of glucosylceramidase requires the presence of a negatively charged phospholipid such as phosphatidylserine (PS) (5, 19). In earlier studies, we provided evidence that Sap C was able to favor the association of glucosylceramidase with PS membranes and that the physical characteristics of PS vesicles markedly influenced their interaction with glucosylceramidase (20, 21).

Most reports dealing with the reconstitution of glucosylceramidase activity by saposins have involved enzyme assays in

‡ To whom correspondence should be addressed: Tel.: 39-6-49902416; Fax: 39-6-49387149; E-mail: avaccaro@net.iss.it.

1 The abbreviations used are: Sap, saposin; MU-Glc, 4-methylumbelliferyl-β-D-glucopyranoside; chol, cholesterol; PS, phosphatidylserine; PC, phosphatidylycholine; LUV, large unilamellar vesicles; SUV, small unilamellar vesicles; TES, 2-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]aminoethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxy-methyl)ethyl]glycine.

This paper is available online at http://www.jbc.org

16862 This paper is available online at http://www.jbc.org
which the substrate was an artificial water-soluble compound, 4-methylumbelliferyl-β-D-glucopyranoside (MU-Glc) (17, 18, 20). When the characteristics of glucosylceramidase activity were investigated with the lipid substrate glucosylceramide inserted into sonicated small unilamellar vesicles (SUV), high activity was achieved upon addition of anionic phospholipids to the lipid bilayer (22, 23). The rate of glucosylceramide degradation depended on the binding of glucosylceramide to SUV, and the presence of acidic phospholipids in the vesicles promoted enzyme binding even in the absence of saposins (23). Since at least Sap C is essential in vivo for the enzymatic degradation of glucosylceramide (13), the question arises whether small liposomes are a reliable membrane model for investigations on the involvement of saposins in sphingolipid hydrolysis. It is well known that the lipid packing in small vesicles is not optimal (24, 25); the lipid surface disorder might facilitate the insertion of glucosylceramidase into the bilayer and obviate the requirement for saposins.

To have a better insight into the molecular mechanism of glucosylceramide degradation and the interactions involved, we have compared the characteristics of the hydrolysis of the sphingolipid inserted either in large or small vesicles. The effects of saposins on the catalytic process and their mode of action have been investigated.

EXPERIMENTAL PROCEDURES

Materials—Glucosylceramide, purified from spleen of patients with Gaucher's disease, was labeled with tritium in the glucose moiety according to McMaster and Radin (26). Phosphatidylcholine (PC) from egg yolk and PS from bovine brain were from Avanti Polar Lipids, Inc. (Alabaster, AL). t-t-dipalmitoyl [dipalmitoyl-1-14C]-PC (110 mCi/mmol) was from NEN Research Products, DuPont de Nemours (Germany). Cholesterol (cho) and MU-Glc were from Sigma. All other chemicals were of the purest available grade.

Saposins A, B, C, and D Preparation—Saposins B, C, and D were purified from spleens of patients with type 1 Gaucher's disease following a procedure previously reported (27); it consisted of heat treatment of a membrane fraction obtained from Avanti's m-14C-cholesterol containing (100 μg total lipid) in 0.1 ml of buffer A, pH 5.0 (10 mM acetate, 150 mM NaCl, 1 mM EDTA). The mixture was then centrifuged with a 42.2 Ti rotor (Beckman) in polycarbonate centrifuge tubes (7 x 20 mm) at 80,000 × g for 1 h. More than 95% of vesicles were found in the pellet as determined by radioactivity measurements. Conversely, in control experiments without liposomes, the saposins were not found to sediment during the ultracentrifugation. After separation of the supernatant, the pellet vesicles were rinsed once with 0.2 ml of buffer A and finally resuspended in 0.2 ml of the same buffer. Saposins in the initial supernatant (free saposins) and in the resuspended vesicles (liposome-bound saposins) were precipitated by addition of 20 μg of albumin and 2 ml of cold acetone. After 2 h at −20 °C, the precipitated saposins were collected by centrifugation, solubilized with 0.1 ml of electrophoresis sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting (below).

To distinguish glycosylceramidase from glucosylceramide synthase, the enzyme (1000 units) was incubated at 37 °C for 15 min with LUV (100 μg total lipid), and the indicated amounts of saposins in 0.2 ml of buffer A, pH 4.5, were added with 10 mM diethioerythritol and 20 μg of albumin. The mixture was centrifuged as above. The amount of glucosylceramidase in the supernatant was determined by measuring the enzyme activity according to the method described. The amount of liposome-bound glucosylceramidase was expressed relative to the amount of enzyme in the supernatant of a sample centrifuged in the absence of liposomes.

RESULTS

Influence of Liposome Size on the Enzymatic Hydrolysis of Liposomal Glucosylceramide—We have previously shown that PS LUV, as opposed to PS SUV, are unable to fully stimulate glucosylceramidase activity toward the artificial substrate MU-Glc (21). Here, we have investigated whether the liposome size has a similar effect on the enzyme activity toward glucosylceramide, the physiological enzyme substrate, inserted in vesicles containing, besides glucosylceramide (5%), cholesterol, PC, and an acidic phospholipid such as PS. Large and small liposomes of identical composition were utilized. As shown in Fig. 1, glucosylceramidase at acidic pH values was able to fully degrade the sphingolipid contained in SUV but not that in LUV; glucosylceramidase activity measured with the LUV system was only 10–15% of the activity measured with the SUV system. This same pattern was observed when the percentage of the sphingolipid in the bilayer was increased from 5 to 10% (data not shown). To reduce changes in the surface of vesicles,
Effect of Saposins on Glucosylceramide Hydrolysis

The ability of the four saposins to activate the enzymatic hydrolysis of liposomal glucosylceramide was investigated. Glucosylceramidase was incubated with glucosylceramide-containing LUV and SUV, in buffer A, adjusted to the indicated pH values. Both LUV and SUV were composed of chol:PC:PS:glucosylceramide (25:50:20:5). The glucosylceramide hydrolysis was measured as reported under “Experimental Procedures.” The deviation for all samples was less than ± 5% of the corresponding mean value.

The optimum pH for the sphingolipid degradation was the same both in the presence and in the absence of saposins, namely pH 5.0 for the SUV system and pH 4.5 for the LUV system.

To check the importance of PS for glucosylceramidase stimulation by Sap A and Sap C, the enzyme assays were repeated utilizing glucosylceramide-containing LUV devoid of PS. Under these conditions, the enzyme activity decreased dramatically, even in the presence of both saposins (Fig. 3A).

The effect of Sap A on the glucosylceramidase hydrolysis observed in this paper was unexpected; in fact, we had previously found that the hydrolysis of the water-soluble enzyme substrate, MU-Glc, was unaffected by Sap A (32). Fig. 3B confirms that, in the presence of PS-containing LUV, Sap A poorly activates the MU-Glc hydrolysis. The enzyme activity toward this substrate is fully stimulated by Sap C, independently of Sap A. It is thus evident that the Sap A stimulation is specific for glucosylceramidase hydrolysis.

Binding Properties of Sap A, Sap C, and Glucosylceramidase—We recently found that Sap A, as opposed to Sap C, fails to bind tightly to phospholipid membranes (32). Since Sap A, especially in combination with Sap C, is able to promote the degradation of glucosylceramide inserted in LUV, we considered the possibility that the sphingolipid and/or Sap C could increase the affinity of Sap A for the lipid surface. Sap A, either alone or in admixture with Sap C, was thus incubated with PS-containing LUV with or without glucosylceramide; the LUV-associated saposins were then separated from nonbound saposins by ultracentrifugation. The identity of the saposins present in the supernatant and in the pellet was evaluated by immunoblotting with specific anti-Sap A or anti-Sap C antibodies. Fig. 4 shows that Sap A poorly associates with the vesicles (compare lanes 2 and 4 with lanes 3 and 5), even in the presence of glucosylceramide (Fig. 4A) and Sap C. Centrifugation separated the two combined proteins; most of Sap A was recovered in the supernatant while most of Sap C was bound to the vesicles either in the presence or in the absence of the sphingolipid (Fig. 4, A and B, lane 5). These results clearly show that the binding properties of Sap A and Sap C are quite different, irrespective of whether the phospholipid membranes contain

---

**FIG. 1.** Effect of liposome size on the enzymatic hydrolysis of liposomal glucosylceramide. Glucosylceramidase was incubated with glucosylceramide-containing LUV (○) or SUV (●), in buffer A, adjusted to the indicated pH values. Both LUV and SUV were composed of chol:PC:PS:glucosylceramide (25:50:20:5). The glucosylceramide hydrolysis was measured as reported under “Experimental Procedures.” The deviation for all samples was less than ± 5% of the corresponding mean value.

**FIG. 2.** Effect of saposins on the enzymatic hydrolysis of glucosylceramide inserted in LUV. Glucosylceramidase was incubated with glucosylceramide-containing LUV in buffer A adjusted to pH 4.5. The composition of LUV and the enzymatic assay were as in Fig. 1. A, increasing amounts of either Sap A (○—○), Sap B (○—△), Sap C (○—□), or Sap D (○—■) were added to the assay. B, the enzyme assay contained a fixed amount of Sap A (5 μM), increasing amounts of Sap C (■—■), or a fixed amount of Sap C (5 μM) and increasing amounts of Sap A (●—●). The points represent means of at least three different experiments. The deviation for all samples was less than ± 5% of the corresponding mean value.

---

**FIG. 3.** Effect of saposins on the enzymatic hydrolysis of glucosylceramide inserted in LUV. Glucosylceramidase was incubated with glucosylceramide-containing LUV in buffer A adjusted to pH 4.5. The composition of LUV and the enzymatic assay were as in Fig. 1. A, increasing amounts of either Sap A (○—○), Sap B (○—△), Sap C (○—□), or Sap D (○—■) were added to the assay. B, the enzyme assay contained a fixed amount of Sap A (5 μM), increasing amounts of Sap C (■—■), or a fixed amount of Sap C (5 μM) and increasing amounts of Sap A (●—●). The points represent means of at least three different experiments. The deviation for all samples was less than ± 5% of the corresponding mean value.
Effect of Saposins on Glucosylceramide Hydrolysis

The purpose of this study was to evaluate the involvement of saposins in the enzymatic degradation of glucosylceramide and to consider the mechanism whereby saposins and lipids may modulate glucosylceramide activity toward its natural substrate. Glucosylceramide was inserted in LUV having the lipid composition estimated to be characteristic of most membranes, namely containing cholesterol and phospholipids (1:3). In many biological studies, the average size of the liposomes used as the model of biological membranes is a critical parameter for the interpretation of the results. In the present research, we have found that glucosylceramide can be degraded more efficiently when inserted in PS-containing SUV rather than in PS-containing LUV. This result stresses the importance of the assay system chosen for the study of glucosylceramidase activity and properties. The different performance of the LUV and SUV systems can only be explained on the basis of lipid organization. In fact, there were no compositional differences between vesicles. Probably, the loose packing of the lipids in the outer surface of small vesicles favors the enzyme interaction with glucosylceramide. The influence of the bilayer curvature on glucosylceramidase activity might have a physiological relevance also on consideration that cerebrosides, such as the glucosylceramide substrate, are possibly involved in the formation and maintenance of highly curved membranes (33).

When glucosylceramide is contained in LUV, where the lipid packing arrangement approaches that of a planar bilayer, glucosylceramidase poorly interacts with its natural substrate unless two saposins, Sap C and A, are added to the liposomal system. Each of the two saposins by itself has a partial effect;
only in admixture do they increase the rate of glucosylceramide hydrolysis up to a level comparable with that observed in the SUV system. The combined effects of the two saposins were always greater than the sum of the stimulation induced by the two saposins separately. Such synergism suggests a different mode of action of Saps A and C. The idea that the two saposins act through different mechanisms is strongly supported by their distinct effects on the glucosylceramidase activity toward two substrates; Sap A stimulates the hydrolysis of glucosylceramide, but not that of the artificial substrate MU-Glc, while Sap C stimulates the hydrolysis of MU-Glc more than that of glucosylceramide.

We previously showed that Sap C has the greatest membrane affinity of the four saposins (32). An acid-induced increased hydrophobicity has been demonstrated as the first step in the Sap C association with membranes (32). After interaction with Sap C, PS-containing LUV acquire the capacity to bind glucosylceramidase (20). The present observation that the Sap C-mediated adhesion of glucosylceramidase to lipid bilayers results in only a partial stimulation of glucosylceramidase hydrolysis indicates that additional factors are required for an optimal interaction between the enzyme and its lipid substrate. The presence of Sap A appears to fulfill this requirement.

Differently from Sap C, Sap A binds poorly to bilayers (present work, and Ref. 32). In parallel, Sap A neither has a significant effect by itself on the interaction of the enzyme with membranes nor potentiates the Sap C capacity to promote this interaction. To explain the stimulation of glucosylceramidase hydrolysis by Sap A, a mechanism different from that of Sap C can be envisaged. As proposed by other groups (16, 17), Sap A might form a complex with glucosylceramidase, causing a conformational change in the structure of the enzyme that results in increased catalysis. In this context, the role of Sap A would be mainly related to the enzyme–glucosylceramide interaction while that of Sap C to the membrane localization of the enzyme.

In general, the activity of membrane-bound enzymes, such as glucosylceramidase, is modulated by their lipid environment. Glucosylceramidase has a strong affinity for acidic phospholipids such as PS (5); these lipids are essential for the binding of glucosylceramidase to liposomes (23, 29) and for the reconstitution of the enzyme activity either in the presence or absence of saposins (present paper, and Refs. 5 and 20). The presence of PS markedly increases also the extent of Sap C binding and the consequent perturbation of the bilayer structure (32). Thus, the Sap C stimulation of glucosylceramidase is possibly related to the Sap C-induced destabilization of membranes, which might favor the interaction of the enzyme with PS. Our past (29, 32) and present results suggest that a change in PS content might regulate the amount of Sap C and glucosylceramidase in lysosomal membranes, allowing acidic phospholipids to have a dynamic function in the glucosylceramide degradation.

In contrast with our findings, some authors have claimed that Sap C does not interact with acidic phospholipids and that this saposin stimulates glucosylceramidase by binding directly to the enzyme (34). The two groups who previously tested the effect of the simultaneous addition of Saps A and C on the glucosylceramidase activity utilized MU-Glc but not glucosylceramide as substrate; under these conditions, they found that Sap A, although being able by itself to activate glucosylceramidase, had no additive effect over the optimal catalytic rate achieved by saturating amounts of Sap C (18, 34). The authors concluded that Sap A and Sap C have the same mode of action and that the two saposins compete for a binding site on glucosylceramidase (18, 34). In our experience, it is critically important to use appropriate conditions for testing the effects of saposins. We could evidence the additivity of the Sap A and Sap C effects only when the degradation of glucosylceramide was measured (see Fig. 3). We did not observe any Sap A stimulation of the MU-Glc hydrolysis, a finding in agreement with Kondoh et al. (35).

When Sap C is absent in consequence of a genetic defect, the accumulation of intralysosomal glucosylceramide and a Gaucher-like disease have been observed (13). In this variant of Gaucher’s disease, glucosylceramidase and Sap A are present at almost normal levels. For authors who believe that Sap A and Sap C act through the same mechanism, this observation argues against a possible physiological function of Sap A as a glucosylceramidase activator (18). Theoretically, if the two saposins have similar properties, a normal Sap A might accomplish the function of a deficient Sap C. By contrast, if the two saposins have different mechanisms of action, as our results indicate, the glucosylceramide accumulation caused by the absence of Sap C might not be prevented by the presence of Sap A.

In conclusion, our work provides new information regarding the influence of saposins on glucosylceramide degradation. According to our findings, glucosylceramidase activity toward its lipid substrate can be modulated by several factors, the more important being the presence of two saposins, Sap C and Sap A, and the membrane structure and composition. The fact that Sap A and Sap C can synergistically affect glucosylceramide hydrolysis suggests the possibility that saposins might accomplish their physiological function not only as single proteins but also in conjunction with each other.

Acknowledgments—The authors thank E. Raia for technical assistance.

REFERENCES

1. O’Brien, J., and Kishimoto, Y. (1991) FASEB J. 5, 301–308
2. Kishimoto, Y., Hiraoka, M., and O’Brien, J. S. (1992) J. Lipid Res. 33, 1255–1267
3. Forst, W., and Sandhoff, K. (1992) Biochim. Biophys. Acta 1126, 1–16
4. Sandhoff, K., Harzer, K., and Forst, W. (1995) In The Metabolic and Molecular Bases of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) pp. 2427–2441, McGraw-Hill Book Co., New York
5. Grew, R. H., Basu, A., La Marco, K., and Prence, E. (1988) Lab. Invest. 58, 5–25
6. Van Dongen, J. M., Willemsen, K., Ginn, E. I., Sips, H. J., Tager, J. M., Barranger, J. A., and Reuser, A. J. J. (1985) Eur. J. Cell Biol. 39, 179–189
7. Paton, B. C., Hughes, J. L., Harzer, K., and Poulos, A. (1989) Eur. J. Cell Biol. 51, 157–164
8. Fujibayashi, S., and Werner, D. A. (1986) J. Biol. Chem. 261, 15339–15343
9. Fujibayashi, S., and Werner, D. A. (1986) Biochim. Biophys. Acta 875, 554–562
10. O’Brien, J. S., Kretz, K. A., Dewji, N., Werner, D. A., Esch, F., and Fuharty, A. (1988) Science 241, 1096–1101
11. Schnabel, D., Schroder, M., Forst, W., Klein, A., Hurwitz, R., Zenk, T., Weber, J., Harzer, K., Paton, B. C., Poulos, A., Suzuki, K., and Sandhoff, K. (1992) J. Biol. Chem. 267, 3312–3315
12. Bradvova, V., Smid, F., Ulrich-Bott, W., Roggendorf, W., Paton, B. C., and Harzer, K. (1993) Hum. Genet. 92, 143–152
13. Christomanou, H., Chahine, P., Pampoulis, T., and Guardiola, A. (1989) Klin. Wochenschr. 67, 999–1003
14. Wenger, D. A., De Gala, G., Williams, C., Taylor, H. A., Stewartson, R. E., Pruitt, J. R., Miller, J., Garen, P. D., and Balentine, J. D. (1989) Am. J. Med. Genet. 34, 255–265
15. Schlote, W., Harzer, K., Christomanou, H., Paton, B. C., Kurtzmann-Kuhn, B., Schmidt, B., Seeger, J., Beutid, U., Schuster, I., and Langenbeck, U. (1991) Eur. J. Pediatr. 150, 584–591
16. Morimoto, S., Martin, B. M., Yamamoto, Y., Kretz, K. A., O’Brien, J. S., and Kishimoto, Y. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3389–3393
17. Fabbrro, D., and Grabowski, G. A. (1991) J. Biol. Chem. 266, 15021–15027
18. Gi, X., Leonova, T., and Grabowski, G. A. (1994) J. Biol. Chem. 269, 16746–16753
19. Basu, A., Grew, R. H., Daniels, L. B., and Clark, L. S. (1984) J. Biol. Chem. 259, 1714–1719
20. Vacekar, A. M., Tatti, M., Ciaffoni, F., Salvioli, R., Maras, B., and Barca, A. (1993) FEBS Lett. 336, 159–162
21. Vacekar, A. M., Tatti, M., Ciaffoni, F., Salvioli, R., Barca, A., and Rencaoli, P. (1993) Biochim. Biophys. Acta 1149, 55–62
22. Sarmientos, F., Schwarzmann, G., and Kishimoto, K. (1986) Eur. J. Biochem. 160, 527–535
23. Vacekar, A. M., Tatti, M., Salvioli, R., Ciaffoni, F., and Gallozzi, E. (1990) Biochim. Biophys. Acta 1033, 73–79
24. Nis, S., Wilshutz, J., and Bentz, J. (1982) Biochim. Biophys. Acta 688, 275–278
25. Okili, S. (1984) J. Membr. Biol. 77, 265–275
26. McMaster, M. C., Jr., and Radin, N. S. (1977) *J. Labelled Compd. Radiopharm.* 13, 353–357
27. Sano, A., Radin, N. S., Johnson L. L., and Tarr G. E. (1988) *J. Biol. Chem.* 263, 19597–19601
28. Murray, G. J., Youle, R. J., Gandy, S. E., Zirzow, G. C., and Barranger, J. A. (1985) *Anal. Biochem.* 147, 301–310
29. Vaccaro, A. M., Tatti, M., Ciaffoni, F., Salvioli, R., and Roncaioli, P. (1992) *Biochim. Biophys. Acta* 1119, 239–246
30. Mayer, L. D., Hope, M. J., and Cullis, P. R. (1986) *Biochim. Biophys. Acta* 858, 161–168
31. Schagger, H., and Von Jagow, G. (1987) *Anal. Biochem.* 166, 368–379
32. Vaccaro, A. M., Ciaffoni, F., Tatti, M., Salvioli, R., Barca, A., Tognozzi, D., and Scerch C. (1995) *J. Biol. Chem.* 270, 30576–30580
33. Curatolo, W., and Neuringer, L. J. (1986) *J. Biol. Chem.* 261, 17177–17182
34. Morimoto, S., Kishimoto, Y., Tomich, J., Weiler, S., Ohashi, T., Barranger, J. A., Kretz, K. A., and O'Brien, J. S. (1990) *J. Biol. Chem.* 265, 1933–1937
35. Kundoh, K., Hineno, T., Sano, A., and Kakimoto, Y. (1991) *Biochem. Biophys. Res. Commun.* 181, 286–292