Parkinson's disease is the second most common neurodegenerative disorder, and the cause is unknown; however, substantial evidence implicates the aggregation of α-synuclein as a critical factor in the etiology of the disease. α-Synuclein is a relatively abundant brain protein of unknown function, and the purified protein is intrinsically unfolded. The amino acid sequence has seven repeats with an apolipoprotein lipid-binding motif, which are predicted to form amphiphilic helices. We have investigated the interaction of α-synuclein with lipid vesicles of different sizes and properties by monitoring the effects on the conformation of the protein and the kinetics of fibrillation. The nature of the interaction of α-synuclein with vesicles was highly dependent on the phospholipid composition, the ratio of α-synuclein to phospholipid, and the size of the vesicles. The strongest interactions were between α-synuclein vesicles composed of 1,2-dipalmitoyl-sn-glycero-3-phosphate/1,2-dipalmitoyl-sn-glycero-3-phosphocholesterol and 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC-(1-glycerol)/1,2-sn-glycero-3-phosphocholine and 1,2-di-

α-Synuclein is an abundant 140-amino acid neuronal protein of unknown function, which is enriched in the presynaptic terminals of neurons (1, 2). It is also an intrinsically unfolded, or natively unfolded, protein, meaning that in the purified form at neutral pH it lacks an ordered secondary or tertiary structure. α-Synuclein is also the major fibrillar component of Lewy bodies, a pathological hallmark of Parkinson’s disease (3). The molecular basis for the distribution of α-synuclein within neurons, and the formation of Lewy bodies, is not well understood. Recent studies have shown that α-synuclein is associated with membranous compartments in cultured cells and brain tissue (4–6). It has been demonstrated that α-synuclein specifically binds to phospholipids with acidic head groups (7–9). Although most of α-synuclein is found in the free cytosolic fraction in the cell, membrane-bound α-synuclein has been suggested to play an important role in fibril formation (10). Therefore, it is likely that differential affinity for specific phospholipids is responsible for the protein location and perhaps fibril formation.

The N-terminal region (approximately residues 1–95) of α-synuclein contains six 11-amino acid imperfect repeats with a highly conservative hexamer motif (KTKEGV), resulting in a variation in hydrophobicity with a strictly conserved periodicity of 11. Such a periodicity is characteristic of the amphipathic lipid-binding α-helical domains of apolipoproteins (11), which have been extensively studied and assigned to subclasses according to their unique structural and functional properties (12, 13). α-Synuclein shares the defining properties of the class A2 lipid-binding helix, distinguished by clustered basic residues at the polar-apolar interface, positioned ±100° from the center of apolar face, a predominance of lysines relative to arginines among these basic residues, and several glutamate residues at the polar surface (12, 13).

The existing literature on the nature of the interactions of α-synuclein with membranes is somewhat contradictory. Previous reports have indicated that α-synuclein interacts with certain phospholipids, which may transform it into a helical conformation (8, 9, 14). It has been reported that small oligomeric forms of α-synuclein preferentially associated with lipid droplets and cell membranes (15) and that α-synuclein binds preferentially to small unilamellar vesicles (SUVs) containing acidic phospholipids (with the induction of a helical circular dichroism (CD) signal), but not to vesicles with a net neutral charge (9). In contrast, strong binding of α-synuclein to large unilamellar vesicles (LUVs) with either anionic or zwitterionic headgroups has also been reported (16). Membranes have been reported to accelerate the fibrillation of α-synuclein (10), and a recent report suggests that α-synuclein aggregation may occur on membrane surfaces (15) and that membranes preferentially induce α-synuclein oligomers. However, it has also been reported that α-synuclein binds tightly to neutral and anionic membranes and that membranes inhibit fibrillation (16).

Our previous studies have demonstrated that the fibril formation of α-synuclein is mediated by a critical partially folded intermediate (17). Here we report that acidic phospholipids induced the partially folded conformation upon binding to phospholipid vesicles at relatively low lipid concentrations, but an α-helix-rich structure was induced at higher concentrations of lipid. Of particular importance is the observation that the partially folded structure led to fibril formation, whereas the α-helical structure inhibited fibril formation.

**MATERIALS AND METHODS**

Expression and Purification of Protein—Recombinant α-synuclein was expressed in *Escherichia coli* and purified as described previously (17).

---

*This work was supported by Grant NS39985 from The National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Chemistry and Biochemistry, University of California, Santa Cruz, CA 95064. Tel.: 831-459-2744; Fax: 831-459-2935; E-mail: enzyme@cats.ucsc.edu.

‡ The abbreviations used are: SUV, small unilamellar vesicle; LUV, large unilamellar vesicle; ThT, thioflavin T; PA, 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC-(1-glycerol)/1,2-sn-glycero-3-phosphocholine; PG, 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC-(1-glycerol); AFM, atomic force microscopy; EM, electron microscopy.
Materials—Thioglycollate (TThT) was obtained from Sigma. 1,2-Dipalmitoyl-sn-glycero-3-phospho-L-serine (PA), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phospho-RAC (1-glycerol) (PG), dissolved in chloroform, were purchased from Avanti Polar Lipids, Inc.

Preparation of SUVs and LUVs—Sonicated SUVs of PA/PC (molar ratio 1:1), PG/PC (molar ratio 1:1), and PC were prepared as described previously (9). LUVs of PA/PC, PG/PC (molar ratios 1:1), and PC were prepared by 10 cycles of freeze-thaw and extruded through a 0.1-μm polycarbonate membrane. α-Synuclein was added to solutions of the vesicles immediately before CD or kinetics measurements for preparations of non-intravesicular protein and mixed with phospholipid before preparation of SUVs or LUVs to obtain intravesicular protein. Vesicles with intravesicular protein contained α-synuclein both in the lumen of the vesicles as well as in the bulk solution (see Fig. 1).

CD Measurements—Far-UV CD spectra were obtained with an AVIV 60DS spectrophotometer (Lakewood, NJ) in a 0.1-cm path length cell. The concentration of protein was kept at 14 μM with the mass ratio of protein to phospholipid varying from 5:1 to 1:10. The final spectra were obtained by calculating the mean of five individual scans and subtracting the background with samples consisting of buffer and vesicles without protein. The percentage of α-helix was determined as described previously (18).

AFM Measurements of α-Synuclein Aggregation—Aliquots (5 μl) of incubation solution were transferred to freshly cleaved mica. Samples adsorbed on to mica were washed with water to remove unbound protein and dried with a stream of dry N2. AFM images were obtained with an Autoprobe CP Multiple AFM (Park Scientific) in tapping mode. Measurements were carried out using silicon cantilevers with a spring constant of 50 newton/m (Park Scientific) and a resonance frequency of 290–350 kHz.

Kinetic Measurements of α-Synuclein Aggregation—A filtered protein sample (0.22 μm) was treated with 0.001 M NaOH for 15 min, then centrifuged for 15 min at 14,000 rpm to remove any preformed aggregates. Freshly prepared stock solutions with concentrations less than 1.0 mg/ml were used within 1 day. Fibril growth experiments involved incubating 0.5 mg/ml purified protein and SUVs or LUVs with varying ratios of α-synuclein to phospholipid in 20 mM Tris-HCl buffer (pH 7.5), 0.1 M NaCl, containing 20 μM TThT at 37 °C with agitation. Fluorescence intensities were recorded in situ at intervals of 30 min using a fluorescence plate reader (Fluoroskan Ascent, Thermo-Labystems) with excitation at 450 nm and emission at 485 nm. The sigmoidal kinetics curves for fibril formation were analyzed as described previously (19). In control experiments we have shown that the presence of TThT (up to at least 20 μM) has no effect on the kinetics of α-synuclein fibrillation.

RESULTS

Binding of PA/PC SUVs or LUVs Induced α-Helical Structure in α-Synuclein—Preparations of SUVs are relatively homogeneous, with a more curved (smaller radius) surface compared with LUVs, and the SUVs are of similar size to synaptic vesicles. The far-UV CD spectrum of α-synuclein at pH 7.5 is typical of an unfolded protein and has a significant negative molar ellipticity at 198 nm. When α-synuclein was mixed with PA/PC (1:1) vesicles at a mass ratio of 1:1 (protein to PA/PC), the CD spectrum showed a decrease in molar ellipticity at 198 nm, indicating an increase in secondary structure (Table I). When the phospholipid mass ratio was increased to 1:1 α-synuclein/(PA/PC) the CD spectra displayed significant negative molar ellipticity (−12.4 × 10° deg⋅cm²⋅mol⁻¹) at 222 nm, and a large positive molar ellipticity at 195 nm (Fig. 2, indicating formation of substantial α-helix (Table I). When the PA/PC content was increased to 1:5 (α-synuclein/lipid), lipid binding was accompanied by a further increase in helical structure from 32 to 73% as the mass ratio of lipid increased from 1:1 to 1:5.

Fig. 2 shows that helix was induced on binding to the acidic phospholipid vesicles regardless of vesicle size (SUVs or LUVs). However, the amount of helix induced was less for LUVs than for the corresponding SUVs (Table I). Negatively stained preparations examined by EM indicated that the large unilamellar vesicles of LUVs are higher than those of SUVs. We used CD to determine whether there were differences in the interaction of α-synuclein with lipids between the inside and outside of the vesicle, we prepared small (SUVs) and larger vesicles (LUVs) with protein trapped inside the vesicles. Although it is hard to estimate the encapsulation efficiencies of α-synuclein in lipid vesicles without separating free α-synuclein from bound protein, it is well known that the encapsulation efficiencies of LUVs are higher than those of SUVs. We used CD to

Table I

| Vesicle size | Protein/lipid (mass ratio) | Intravesicular protein | α-Helical content | Lag time |
|-------------|---------------------------|------------------------|-------------------|---------|
| SUV         | 5:1                       | –                      | 9                 | 4.5 ± 0.7 |
|             | +                         | 29                     | 6.8 ± 0.8         |         |
|             | 1:1                       | –                      | 32                | 12.2 ± 1.6 |
|             | +                         | 58                     | 18.5 ± 1.9        |         |
|             | 1:5                       | –                      | 73                | ∞        |
|             | +                         | 84                     | ∞                 |         |
| LUV         | 5:1                       | –                      | 8                 | 8.8 ± 0.6 |
|             | +                         | 8                      | 7.5 ± 0.9         |         |
|             | 1:1                       | –                      | 17                | 17.3 ± 1.5 |
|             | +                         | 17.7                   | 19.3 ± 2.1        |         |
|             | 1:5                       | –                      | 61.2              | 29.2 ± 3.8 |
|             | +                         | 69.5                   | 39.8 ± 4.5        |         |
examine the conformational changes, with the results shown in Fig. 2, while the α-helix content is given in Table I. There was consistently more helix induced with the vesicles containing intravesicular α-synuclein, but the effect with LUVs was smaller. Thus, the helix content increased from 73 to 84% for SUVs, and from 61 to 70% for LUVs, when the protein was present inside the vesicle lumen. At low protein to lipid ratios the amount of helix was more than doubled with the SUVs, consistent with greater induction of helix to the more curved interior surface.

To determine whether the tendency to induce α-helix is a general property of phospholipids with acidic head groups, PG/PC, PS/PC, and PC alone were also prepared and CD spectra were collected. An equivalent phenomenon was observed when negatively charged PA/PC was replaced by negatively charged PG/PC or PS/PC (data not shown) to form SUVs or LUVs. However, binding to neutral lipid vesicles of PC only slightly decreased the ellipticity at 198 nm (Fig. 4), and no helical structure was observed.

Fibrillation of α-Synuclein Modulated by Phospholipid Vesicles—α-Synuclein was incubated with small and large PA/PC vesicles with various concentrations of protein and lipid. These studies involved incubation of vesicles at 37 °C for 3 days. EM images demonstrated that the basic structure of the vesicles did not change (Fig. 3). Fig. 4A shows the effect on the kinetics of α-synuclein fibrillation monitored by ThT fluorescence of the addition of 0.5 mg/ml α-synuclein to the vesicles, together with a control solution in which α-synuclein was added to the buffer in the absence of vesicles. ThT is a fluorescence dye that binds to amyloid fibrils leading to a large increase in the fluorescence intensity. The fibrillation of α-synuclein was shown by the increase in ThT signal and then verified by EM and AFM images.

We found that the fibrillation of α-synuclein was affected by

PA/PC vesicles in a concentration and size dependent fashion. With moderate concentrations of PA/PC, which induced a partially folded α-synuclein intermediate, as evidenced by the CD spectra (Fig. 2), the rate of fibril formation of α-synuclein was accelerated, regardless of vesicle size (Fig. 5). EM and AFM images show the size and shape of fibrils formed in the presence of PA/PC (Fig. 6); the fibrils had a typical height of 7.6 nm and 28-nm periodic left-handed twisted structure. This morphology is very similar to that of fibrils grown in lipid-free solution, indicating a similar fibrillation pathway as in the absence of lipid.

Increasing the relative PA/PC concentration in the incubation solution led to a decrease in rate of fibril formation, as shown by the increased lag time (Fig. 5). Under these conditions, CD spectra also showed a change in secondary structure from partially unfolded intermediate to mostly helical structure. There was no significant difference in the kinetics of fibrillation between SUVs containing intravesicular protein or non-intravesicular protein.

When the mass ratio of protein to lipid was increased from 5:1 to 1:5, the helical structure content increased from 7–8 to 60–70% for LUV-bound α-synuclein. Concurrently, the lag time for fibrillation increased from 8.5 ± 0.6 h to 29.2 ± 3.8 h for non-intravesicular LUVs and from 7.5 ± 0.9 to 39.8 ± 4.5 h for intravesicular protein in the LUVs, correlating well with the increased helix content (Table I). For the SUV-bound α-synuclein, the helix content increased to 70–80%, and fibril formation was completely inhibited (Fig. 5, A and B). No fibrils were formed under these conditions in 4 weeks. In control experiments with no lipid, fibrils formed in 14 h. Similar re-
Results were obtained with vesicles of PG/PC and PS/PC (data not shown).

PC vesicles, with their neutral head groups, were examined to address the possible effects of charge and crowding on protein association and fibrillation. PC vesicles had no effect on the fibrillation of α-synuclein when the protein to lipid ratio was increased up to 1:10, as shown in Fig. 4B. This demonstrates that fibril formation was inhibited by the α-helical conformation and not by the presence of the vesicles themselves.

DISCUSSION

Our previous results demonstrated that fibril formation of α-synuclein was initiated by a conformational change from the natively unfolded structure to a partially folded, β-sheet-containing conformation (17). The current investigation focused on fibril formation by membrane-associated α-synuclein. We used circular dichroism to estimate the secondary structure and thioflavin T assays to monitor the fibril formation, and the results were compared with lipid-free protein. The local concentration of protein is significantly increased upon binding to the lipid vesicles (20).

Interestingly, the effects of the vesicles on α-synuclein fibrillation were very dependent on the ratio of lipid to protein (under all conditions there was a molar excess of lipid). For example, at low mass ratios of PA/PC vesicles to α-synuclein, there was a substantial increase in rate of fibrillation, as manifested by the decrease in lag time from 14 ± 2 h for lipid-free protein to 4.5 ± 0.7 h for lipid-bound (SUVs) α-synuclein. This acceleration of fibrillation coincided with a change in the secondary structure of α-synuclein from natively unfolded to that of the partially folded intermediate. We attribute this increase in fibrillation rate to the formation of the partially folded intermediate conformation. In contrast, when the lipid content increased, the α-synuclein conformation changed to 60–85% α-helix for both SUVs and LUVs of PA/PC (and PG/PC and PS/PC). Since no fibrils were formed under these conditions, we conclude that the helical conformation prevents formation of fibrils. This observation may be of major physiological significance; if under normal conditions α-synuclein is predominantly bound to membranes in dopaminergic neurons, this would minimize its chances of aggregation. On the other hand, factors leading to significant reduction in the membrane-bound form of α-synuclein may lead to aggregation, which may contribute to neurodegeneration.

FIG. 4. PC vesicles do not induce helical structure in α-synuclein and inhibit fibrillation. A, kinetics of α-synuclein fibrillation monitored by thioflavin T fluorescence. Circles, α-synuclein alone; inverted triangles, fibrillation in the presence of PC SUVs at protein:lipid mass ratios of 1:5, 1:10 (squares), and 1:20 (diamonds). A small amount of inhibition is observed in the presence of the PC vesicles. B, far-UV CD spectra of α-synuclein (solid line) binding to PC SUVs at protein:lipid mass ratios of 5:1 (dotted line), 1:1 (dashed line), and 1:5 (dash-dotted line).

FIG. 5. The effect of vesicles on the kinetics of α-synuclein fibrillation. Fibrillation was monitored by thioflavin T fluorescence. Circles, α-synuclein alone. A, in the presence of PA/PC vesicles in the form of non-intravesicular protein SUVs; B, intravesicular protein SUVs; C, non-intravesicular protein LUVs; D, intravesicular protein LUVs at protein:lipid ratios of 5:1 (inverted triangles), 1:1 (squares), and 1:5 (diamonds). High concentrations of SUVs completely inhibited fibrillation (A and B).

FIG. 6. The morphology of α-synuclein fibrils grown in the presence of vesicles is similar to that of fibrils grown in the absence of lipid. Left panel, EM image of fibrils; right panel, AFM image of an individual α-synuclein fibril in the presence of PA/PC SUVs at a protein:lipid ratio of 1:1.
α-synuclein could result in pathological effects emanating from the aggregation of α-synuclein.

The effect of the vesicles on the fibrillation rate depends on the vesicle size. In the case of SUVs, regardless of whether or not the protein was in the vesicle core (lumen), fibril formation is inhibited completely if the helix content is above 70%. However, with LUV-bound α-synuclein the rate of fibrillation was decreased, but not totally inhibited, with 60 to 70% α-synuclein in a helical conformation. The most likely explanation is that less α-synuclein is bound to the LUVs compared with the SUVs (at the same lipid/protein mass ratios), and thus some α-synuclein is not bound to the vesicles and is present in the partial folded conformation and goes on to form fibrils. This may be a reflection of the increased lipid cooperativity of LUVs compared with SUVs.

On the other hand, with intravesicular protein, α-synuclein can become locally concentrated within a single vesicle and form fibrils in the core (lumen). We have demonstrated that fibrillar α-synuclein will disrupt lipid membranes in a short time.2 Agitation in the presence of a Teflon bead led to further destruction of the membrane. Thus the initially formed fibrils in the vesicle core can serve as seeds for further fibrillation after they are released to the solution outside the vesicles. This is confirmed by the observation of shorter lag times for intravesicular protein in LUVs as opposed to intravesicular protein in SUVs (Fig. 5).

We have previously shown that formation of the α-synuclein partially folded intermediate leads to fibrillation, presumably due to self-association driven by regions of exposed hydrophobic residues (17, 21–23). However, when α-synuclein is in its helical conformation it preferentially binds to lipid. Thus, exposure of α-synuclein to lipid vesicles leads to preferential binding to the membrane and induction of helix, probably simultaneously, and prevention of protein self-association. Therefore, the protein-lipid interaction induces and stabilizes the helical structure and thus prevents aggregation.

Molecular crowding has been shown to dramatically stimulate the fibrillation of α-synuclein (24). Thus it is possible that there may be some crowding effects on α-synuclein fibrillation at high lipid vesicle concentrations. To determine whether this was a contributing factor, we used PC vesicles, which have been reported not to bind α-synuclein (9). These vesicles had minimal effect on the conformation of α-synuclein, presumably reflecting minimal binding to the vesicles containing neutral head groups and no evidence for helix formation was observed.

The lag times for fibrillation, estimated from kinetics curves shown in Fig. 5, increase slightly from 14 ± 2 h to 15 ± 2 (5:1), 17 ± 2 (1:1), and 19 ± 2 (1:5) h in a lipid concentration-dependent fashion. This slight inhibition of fibrillation is probably due to some interaction with the vesicles. The complete inhibition of fibril formation by PA/PC SUV-bound α-synuclein thus results from the high helical structure and not molecular crowding. This is also verified by the observation that LUVs with the same amount of lipid, and thus the same crowding conditions, result in only moderate fibril inhibition.

We therefore conclude that the α-helical conformation of α-synuclein does not fibrillate. This is also in agreement with investigations of the effect of the osmolyte trimethylamine N-oxide, which show that high concentrations of trimethylamine N-oxide induce a helical conformation in α-synuclein, and a corresponding lack of fibrillation (21).

REFERENCES

1. Maroteaux, L., and Scheller, R. H. (1991) Brain Res. Mol. Brain Res. 11, 335–343
2. Maroteaux, L., Campanelli, J. T., and Scheller, R. H. (1988) J. Neurosci. 8, 2804–2815
3. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 6469–6473
4. George, J. M., Jin, H., Woods, W. S., and Clayton, D. F. (1995) Neuron 15, 361–372
5. McLean, P. J., Kawamata, H., Rübel, S., and Hyman, B. T. (2000) J. Biol. Chem. 275, 8812–8816
6. Irizarry, M. C., Kim, T. W., McNamara, M., Tanzi, R. E., George, J. M., Clayton, D. F., and Hyman, B. T. (1996) J. Neuropathol. Exp. Neurol. 55, 889–895
7. Perrin, R. J., Woods, W. S., Clayton, D. F., and George, J. M. (2000) J. Biol. Chem. 275, 34393–34398
8. Jo, E., McLaurin, J., Yip, C. M., St. George-Hyslop, P., and Fraser, P. E. (2000) J. Biol. Chem. 275, 34358–34364
9. Davidson, W. S., Jonas, A., Clayton, D. F., and George, J. M. (1998) J. Biol. Chem. 273, 34435–34449
10. Lee, H. J., Choi, C., and Lee, S. J. (2001) J. Biol. Chem. 277, 671–678
11. Clayton, D. F., and George, J. M. (1999) Trends Neurosci. 21, 249–254
12. Segrest, J. P., De Loof, H., Dohlman, J. G., Bourette, C. G., and Anantharamiah, G. M. (1990) Proteins 8, 103–117
13. Segrest, J. P., Jones, K. M., De Loof, H., Brouillet, C. G., and Venkataraman, Y. V., and Anantharamiah, G. M. (1992) J. Lipid Res. 33, 141–166
14. Eliezer, D., Kutluay, E., Bussell, R., Jr., and Browne, G. (2001) J. Mol. Biol. 307, 1061–1073
15. Cole, N. B., Murphy, D. D., Grider, T., Ruster, S., Brasaemle, D., and Nussbaum, R. L. (2002) J. Biol. Chem. 277, 6344–6352
16. Narayan, V., and Scarlata, S. (2001) Biochemistry 40, 9927–9934
17. Uversky, V. N., Li, J., and Fink, A. L. (1998) J. Biol. Chem. 273, 10737–10744
18. Chen, Y. H., Yang, J. T., and Martinez, H. M. (1972) J. Biol. Chem. 247, 953–959
19. Nielsen, L., Frukaer, S., Drange, J., Uversky, V. N., and Fink, A. L. (2001) Biochemistry 40, 8397–8409
20. Walde, P., and Ichikawa, S. (2001) Biomol. Eng. 18, 143–177
21. Uversky, V. N., Li, J., and Fink, A. L. (2001) FEBS Lett. 509, 31–35
22. Uversky, V. N., Li, J., and Fink, A. L. (2001) FEBS Lett. 505, 105–108
23. Uversky, V. N., Li, J., and Fink, A. L. (2001) FEBS Lett. 515, 44284–44296
24. Uversky, V. N., Cooper, M., Bower, K. S., Li, J., and Fink, A. L. (2002) FEBS Lett. 515, 99–103

2 M. Zhu, J. Li, and A. Fink, manuscript in preparation.
Lipid Binding Inhibits α-Synuclein Fibril Formation
Min Zhu and Anthony L. Fink

J. Biol. Chem. 2003, 278:16873-16877.
doi: 10.1074/jbc.M210136200 originally published online March 5, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M210136200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 24 references, 10 of which can be accessed free at
http://www.jbc.org/content/278/19/16873.full.html#ref-list-1