Phosphorylation of the Active, A_1 Component of Cholera Toxin by Protein Kinase†

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ABSTRACT: Cholera toxin, an activator of adenylate cyclase in a wide variety of cells, is a substrate for the phosphotransferase reaction catalyzed by purified cyclic adenosine 5′-monophosphate dependent bovine cardiac muscle protein kinase and the protein kinase associated with human erythrocyte membranes. Phosphorylation occurs when the toxin is dissociated with 5–20 mM dithiothreitol and is restricted to the A_1 or "adenylate cyclase activating" subunit of the toxin.

Cholera toxin stimulates cyclic AMP[1] synthesis in a variety of cells. Unlike polypeptide hormones, it activates adenylate cyclase only after an appreciable time lag, and its effect is irreversible (for review, see Finkelstein, 1973; Cuatrecasas, 1973; Holmgren et al., 1973a,b; King and Van Heyningen, 1973; Pierce, 1973; Van Heyningen, 1974). Studies of the chemical structure of cholera toxin (Van Heyningen, 1974; Lo Spalluto and Finkelstein, 1972; Lai et al., 1975) and its plasma membrane receptor, ganglioside GM_1 (Cuatrecasas, 1973; Van Heyningen et al., 1971), and investigations of toxin action in subcellular fractions (Gill, 1975; Gill and King, 1975; Bitensky et al., 1975) have provided a model for the mechanism of action of cholera toxin. The holotoxin, molecular weight, 84 000, is composed of an A subunit (mol wt 28 000) and five or six B subunits (mol wt 9500 each) (Lai et al., 1975). B subunits anchor the toxin to the cell membrane by binding to ganglioside GM_1. This enables the A subunit to interact with either adenylate cyclase or some other molecule which in turn activates the cyclase (Gill and King, 1975; Bennett et al., 1975). The A subunit has two polypeptide chains—A_1, molecular weight 20 000, and A_2, mol wt 7500—linked together by a single disulfide bond (Lai et al., 1975). The ability to activate adenylate cyclase in membrane preparations resides exclusively in the A_1 peptide (Gill, 1975; Gill and King, 1975; Bitensky et al., 1975). In intact cells, the whole toxin molecule is required for activation and little (Van Heyningen and King, 1975) or no (Gill, 1975) activation occurs upon addition of A_1 alone. In cell-free preparations, on the other hand, A_1 activates adenylate cyclase without the lag characteristic of the intact cell system (Gill and King, 1975). Activation by A_1 of adenylate cyclase in membrane preparations requires NAD and possibly other, as yet unidentfied, cytoplasmic factors (Gill, 1975).

The observations that optimal activation of adenylate cyclase in intact cells takes 30–90 min, that the hydrophobic A subunit must be released or at least exposed in order for this activation to occur (Bitensky et al., 1975), and that nondialyzable cytoplasmic factors are important in the activation process all suggest that modification of cholera toxin or some component of it may contribute to its biological activity. Evidence is now presented that cholera toxin can be modified by phosphorylation catalyzed by cAMP-dependent protein kinases from bovine heart and human erythrocyte membranes. Phosphorylation occurs on the A_1, adenylate cyclase activating subunit of the toxin under conditions in which the quaternary structure of the holotoxin is altered by dithiothreitol.

Materials and Methods

The cAMP-dependent protein kinase from bovine cardiac muscle was purified and assayed as previously reported (Rubin et al., 1974). Unless otherwise indicated, only purified preparations (specific activity: 700 nmol min^{-1} mg^{-1} (Rubin et al., 1974)) were used to phosphorylate the toxin. Human erythrocyte membranes were prepared according to Dodge et al. (1963). Polyacrylamide disc gel electrophoresis in 10% acrylamide (referred to in the text as standard polyacrylamide gel electrophoresis) was performed using the procedure of Davis (1964); electrophoresis in 1% sodium dodecyl sulfate and 1% mercaptoethanol was carried out as described by Fairbanks et al. (1971). Following electrophoresis, gels were stained with Coomassie blue, destained in 5% methanol containing 7% glacial acetic acid (Rubin et al., 1972), scanned at an absorbance of 500 nm in a Gilford recorder attached to a Zeiss spectrophotometer, and then sliced into 1-mm slices. Radioactivity in the gel was assayed by placing one or two slices in a vial containing 5 ml of Hydromix (New England Nuclear) and counting in a Packard liquid scintillation spectrometer. Protein was determined by the method of Lowry et al. (1951).

Purified cholera toxin obtained from Schwarz/Mann yielded one protein band on standard 10% polyacrylamide gel electrophoresis. Subunits A and B, prepared by gel filtration on Sephadex G-75 in 5% formic acid, were kindly provided by Dr. C. Y. Lai.

Results

Cardiac muscle protein kinase catalyzes a time-dependent transfer of ^{32}P from [γ-^{32}P]ATP to cholera toxin. The reaction is dependent upon the presence of dithiothreitol (Table I) and proceeds less rapidly with heated than with native toxin (Table I, Figure 1). NAD is unable to substitute for the dithiothreitol requirement, whereas other sulhydryl-containing compounds (e.g., monothioglycerol) are effective. The toxin itself has no phosphotransferase activity.

In various experiments, 0.5–0.8 mol of ^{32}P was incorporated per mole of cholera toxin. Addition of a second aliquot of

† From the Departments of Medicine and Molecular Biology, Albert Einstein College of Medicine, New York, New York 10461. *Received October 30, 1975.* This study was supported by grants from the National Institutes of Health (AM 09038), National Science Foundation (GB 43581), and American Cancer Society (BC-12F).

‡ Recipient of a Career Developmental Award from the United States Public Health Service and a Career Scientist Award from the Irma T. Hirschl Trust.

[1] Abbreviations used are: cAMP, adenosine 3′:5′-monophosphate; NAD, nicotinamide adenine dinucleotide.

TABLE I: Phosphorylation of Cholera Toxin by Protein Kinase.[a]

Additions	Acid Precipitable ^{32}P (cpm)	
	20 min	40 min
Kinase, cAMP, dithiothreitol	532	397
Kinase, cAMP, dithiothreitol, toxin	2337	3572
Heated kinase, cAMP, dithiothreitol, toxin	0	89
Kinase, cAMP, dithiothreitol, heated toxin	694	1596
Kinase, cAMP, NAD, toxin	650	891

[a] Cholera toxin (50 μg) was incubated in 25 μl of 80 mM potassium phosphate buffer, pH 7.1, at 37 °C containing 8 mM $MgSO_4$, 0.02 mM [γ-^{32}P]ATP, 11.9 cpm/pmol, and, where indicated, 40 mM dithiothreitol, 1 μM cAMP, 1.0 mM NAD, and 2 μg of protein kinase. At the indicates times, 1.0 mg of bovine serum albumin was added and reactions were terminated immediately thereafter by adding 1.0 ml of cold 10% trichloroacetic acid. Precipitates were collected on Whatman glass fiber (GF/c) filters, washed with 20 ml of 5% trichloroacetic acid, and dried, and their radioactivity was determined in a low background Nuclear Chicago gas flow counter. Control incubations containing the reaction mixture without kinase or cholera toxin were carried out in parallel and the radioactivity retained by these filters was subtracted from that found in the experimental assays. Where indicated, toxin and protein kinase were heated for 5 min in a boiling water bath.

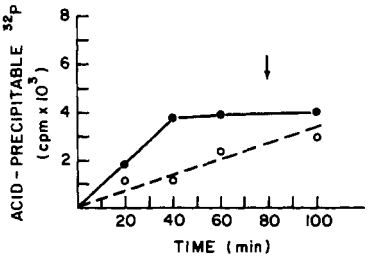

FIGURE 1: Time course for phosphorylation of heated and native cholera toxin. Incubations were at 37 °C in a final volume of 20 μl and contained 25 μg of cholera toxin, 2 μg of protein kinase, 10 mM $MgSO_4$, 0.2 mM [γ-^{32}P]ATP (12 cpm/pmol), 1 μM cAMP, and 50 mM dithiothreitol in 100 mM potassium phosphate buffer, pH 7.1. Controls containing the complete reaction mixture minus cholera toxin were incubated in parallel and their trichloroacetic acid precipitable radioactivity was subtracted from the corresponding experimental values. Cholera toxin was placed in a boiling water bath for 5 min and then cooled at 37 °C. At 80 min (arrow) an additional 2 μg of protein kinase was added to the tube containing native cholera toxin and the reaction allowed to proceed for 20 min. Reactions were terminated and radioactivity was determined as indicated in the legend to Table I. Native cholera toxin (—●—); heated cholera toxin (- -O- -).

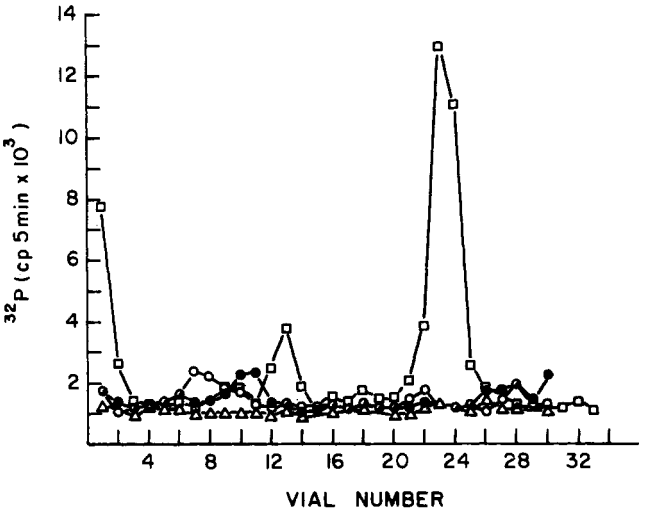

FIGURE 2: Analysis of the ^{32}P-incorporated into cholera toxin by polyacrylamide gel electrophoresis. Cholera toxin (25 μg) was incubated for 40 min at 37 °C in 60 μl of 50 mM potassium phosphate buffer containing, where indicated, 16 mM dithiothreitol, 2 μg of protein kinase, 40 μM [γ-^{32}P]ATP (16 cpm/pmol), 5 mM $MgSO_4$, and 1 μM cAMP. Following incubation, the reaction mixtures were applied directly to 10% polyacrylamide gels and subjected to electrophoresis as described in Methods. The patterns depicted are of incubations that were complete (□) or lacking in either cholera toxin (●), protein kinase (△), or cAMP (○). The recovery of radioactivity on the gels was approximately 50%. The large rapidly moving peak of radioactivity has been designated A_1-phosphate. No phosphorylation of cholera toxin was found when incubations were carried out in the absence of dithiothreitol. The small peaks of radioactivity correspond to the positions of protein kinase and its cAMP-binding protein. In the absence of cAMP, ^{32}P is seen in the position of the holoprotein kinase; in the presence of cAMP, phosphorylation is associated with the cAMP-binding protein component of protein kinase. Self-phosphorylation of protein kinase was not significantly influenced by dithiothreitol.

protein kinase to partially phosphorylated toxin failed to increase the amount of ^{32}P incorporated (0.56 pmol of ^{32}P/pmol of toxin) (Figure 1). Protein-bound ^{32}P is stable in acid and solubilized by heating at 100 °C for 15 min in 1.0 N NaOH. Since phosphorylation is catalyzed by a purified cAMP-dependent protein kinase that only transfers phosphate to serine and threonine residues of specific protein substrates (Rubin et al., 1974), it is likely that one or both of these residues is the phosphate acceptor in the toxin molecule. Each subunit of cholera toxin contains both serine and threonine residues. A_1, A_2, and B subunits contain 13, 7, and 4 mol of serine and 6, 4, and 7 mol of threonine per mole of peptide, respectively (Lai et al., 1975).

During the course of incubation of cholera toxin with either dithiothreitol alone or dithiothreitol, protein kinase, [γ-^{32}P]ATP, and Mg^{2+} in the absence of cAMP, the amount of native cholera toxin diminishes and two new protein bands can be detected by standard polyacrylamide gel electrophoresis. The slowest moving band was shown to be composed of B subunits by subjecting it to electrophoresis directly into a sodium dodecyl sulfate-mercaptoethanol polyacrylamide gel and comparing its mobility with that of purified B in both electrophoretic systems. In analogous experiments, the fastest moving band was found to be A_1. Upon treatment with cAMP-activated protein kinase, an additional protein was observed that appeared only under conditions in which cholera toxin was phosphorylated. The radioactivity in this gel was located in three positions (Figure 2): in protein kinase which catalyzes its own phosphorylation in the presence or absence of dithiothreitol (Erlichman et al., 1974; Rosen and Erlichman, 1975), in the fast moving band which appears upon phosphorylation of cholera toxin and has been designated A_1-phosphate (see below), and at the top of the gel.

Electrophoresis in sodium dodecyl sulfate-mercaptoethanol (Figure 3) or sodium dodecyl sulfate-8 M urea showed that radioactivity other than that associated with the cAMP-binding protein component of the protein kinase was located in a single band corresponding to peptide A_1. Thus, the radioactivity found in the fast moving protein band and at the top of the gel under conditions of standard 10% polyacrylamide gel electrophoresis was associated with a protein whose mobility in sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponded to the purified A_1 subunit (Lai et al., 1975).

Phosphorylation of the A_1 subunit (Figure 2) appears to

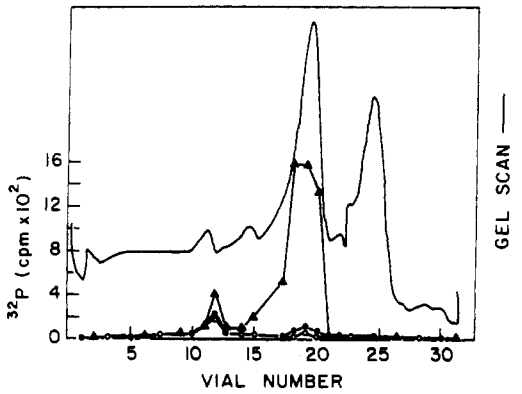

FIGURE 3: Analysis of the ^{32}P incorporated into cholera toxin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cholera toxin (50 μg) was incubated for 40 min at 35 °C in 50 μl of 50 mM potassium phosphate buffer containing 5 mM $MgSO_4$, 80 μM [γ-^{32}]ATP (10 cpm/pmol), 4 μg of protein kinase, and either 1 μM cAMP plus 40 mM dithiothreitol (▲), 40 mM dithiothreitol (●), or 1 μM cAMP (○). Following electrophoresis, staining, and scanning, the gels were sliced and their radioactivity was assayed, as described under Methods. In the scan, the two small peaks are, from left to right, the cAMP-binding protein and the catalytic subunit of the protein kinase. The two large peaks are the A_1 and B subunits of cholera toxin. Radioactivity was found only in the cAMP-binding protein of protein kinase and in the A_1 subunit of cholera toxin.

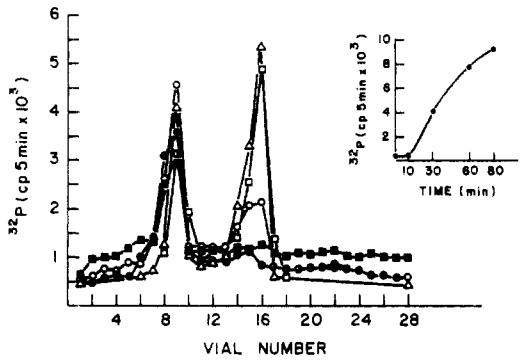

FIGURE 4: Analysis of the time course of phosphorylation of cholera toxin by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Cholera toxin (5 μg) was incubated with 10 mM dithiothreitol, 4 μg of protein kinase, 2 mM $MgSO_4$, 1 μM cAMP and 0.2 mM [γ-^{32}P]ATP (150 cpm/pmol) in 40 μl of 50 mM potassium phosphate buffer, pH 7.1, for 0 (●), 10 (■), 30 (○), 60 (□), and 80 (△) min at 37 °C. Incubations were then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed as described under Methods. Standards of purified A and B subunits were also applied to gels and subjected to electrophoresis in parallel with the experimental samples. The first peak of radioactivity (from left to right) is in the cAMP-binding protein of protein kinase. The second peak corresponds to the position of A_1. The inset depicts an estimate of the total radioactivity associated with the A_1 peak at each time point.

depend upon dissociation of the toxin into A and B subunits by high concentrations of sulhydryl-containing compounds. The presence of dithiothreitol does not affect the protein kinase activity per se and radioactivity was not found in the protein band corresponding to the position of holotoxin (see Figure 2). Phosphorylation occurs more rapidly when toxin is pretreated with dithiothreitol than when the phosphotransferase reaction and the action of dithiothreitol are allowed to proceed concomitantly. When cholera toxin, treated with dithiothreitol for 40 min and then phosphorylated for various periods of time, was followed by electrophoresis in standard polyacrylamide gels, native cholera toxin disappeared and radioactivity accumulated at the top of the gel. Since this radioactivity migrates as A_1 on electrophoresis in sodium dodecyl sulfate, it is probable that the phosphorylated A_1—like purified A or A_1—forms aggregates that are unable to penetrate the 10% acrylamide gel. However, the possibility that some of it may be associated with aggregated phosphorylated holotoxin cannot be excluded. When phosphorylation of cholera toxin was allowed to proceed in the presence of dithiothreitol and samples were removed at various times and subjected to polyacrylamide gel electrophoresis in sodium dodecyl sulfate-mercaptoethanol or sodium dodecyl sulfate-8 M urea, the radioactivity associated with A_1 increased with time (Figure 4). This contrasted with the ^{32}P labeling of the cAMP binding protein component of protein kinase which occurs extremely rapidly (Erlichman et al., 1974).

Phosphorylation of the subunit A_2 was not observed when holotoxin was treated with dithiothreitol in the presence of protein kinase. Since sulfhydryl-containing compounds at concentrations known to dissociate A into A_1 and A_2 are required for the phosphorylation of A_1 from the native toxin, it was not possible to deduce whether unreduced A could serve as a substrate for phosphorylation. To answer this, purified A was tested as a substrate for phosphorylation in the presence and absence of dithiothreitol. Phosphorylation of A was only slightly stimulated by dithiothreitol, whereas phosphorylation of the native cholera toxin was dependent upon its addition. When the subunits treated in this manner were then subjected to standard polyacrylamide gel electrophoresis, all of the ^{32}P transferred to A in the presence of dithiothreitol moved in positions consistent with A_1, that is, the radioactivity was present at the top of the gel and in the fast moving A_1-phosphate band. Some radioactivity was also found close to the gel front. This may have been A_2-phosphate, although A_2-phosphate was not seen when the holotoxin was used as substrate. The ^{32}P transferred to the A subunit in the absence of dithiothreitol remained at the top of the gel, consistent with the position of standard purified A.

Membrane-bound protein kinases also phosphorylate the A_1 subunit of cholera toxin in a dithiothreitol-dependent reaction. When human erythrocyte membranes which contain protein kinase activity (Rubin et al., 1972) were incubated with cholera toxin, dithiothreitol, [γ-^{32}P]ATP, Mg^{2+}, and cAMP, ^{32}P was incorporated into endogenous membrane proteins (Rubin and Rosen, 1973) as well as into a protein that corresponded to the position of the A_1 peptide and was found only in membranes treated with toxin.

Discussion

Soluble cAMP-dependent protein kinase and a membrane-associated kinase are able to catalyze the phosphorylation of the active A_1 peptide of cholera toxin. The stoichiometry of phosphate incorporation catalyzed by bovine heart protein kinase approaches 1 mol of P^{32}/mol of holotoxin treated with high concentrations of dithiothreitol or other sulfhydryl-containing compounds. Low concentrations (2 mM) of dithiothreitol are far less effective than higher concentrations (20-40 mM) in preparing the toxin for substrate activity. One can conclude from studies of the behavior of cholera toxin and its subunits on standard and sodium dodecyl sulfate-mercaptoethanol polyacrylamide gel electrophoresis that dithiothreitol partially dissociates the toxin into its subunits, B, A_1, and, presumably, A_2. The A_2 peptide, however, was not reliably detected in the electrophoretic systems employed. B and A_1 form aggregates which, in the case of A_1, do not enter the 10% polyacrylamide gel and in the case of B migrate very slowly. A small amount of A_1 does penetrate the gel. Upon the addition of an active phosphotransferase system, A_1-phosphate is formed. Some A_1-phosphate moves rapidly into the gel but

most of it probably aggregates and does not penetrate. It is also possible that the A_1-phosphate which moves ahead of A_1 in the 10% polyacrylamide gel is multiply phosphorylated.

We do not know whether phosphorylation plays a role in any aspect of the interaction of cholera toxin with cells. Bourne et al. (1975) have reported that cholera toxin is able to induce the synthesis of cAMP in mouse lymphoma S_{49} cells defective in cAMP-dependent protein kinase. These mutants, however, contain substantial cAMP-independent protein kinase activity. Quite apart from its possible biological function, phosphorylation of cholera toxin may be useful as a probe for studying the subunit interactions of the toxin molecule, for tracking the molecule during the course of its activity in the cell, and for analyzing the sequence around the phosphorylated amino acid for comparison with sequences derived from other kinase substrates.

Acknowledgments

I am indebted to Dr. Mark Bitensky for interesting me in this subject and for sharing ideas and materials. I also thank Dr. C. Y. Lai for permitting me to read his unpublished manuscripts and for gifts of purified subunits, Dr. C. S. Rubin for discussions and for preparations of human erythrocyte membranes, and Mr. Eseng Lai for development of some of the electrophoretic procedures.

References

Bennett, V., Keefe, E. O., and Cuatrecasas, P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 33.

Bitensky, M. W., Wheeler, M. A., Mehta, H., and Miki, N. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2572.

Bourne, H. R., Coffino, P., Melmon, K. C., Tomkins, G. M., and Weinstein, Y. (1975), *Adv. Cyclic Nucleotide Res. 5,* 771.

Cuatrecasas, P. (1973), *Biochemistry 12,* 3547, 3558, 3567, 3577.

Davis, B. (1964), *Ann. N.Y. Acad. Sci. 121,* 404.

Dodge, J. T., Mitchell, C., and Hanahan, D. J. (1963), *Arch. Biochem. Biophys. 100,* 119.

Erlichman, J., Rosenfeld, R., and Rosen, O. M. (1974), *J. Biol. Chem. 249,* 5000.

Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971), *Biochem. J. 13,* 2606.

Finkelstein, R. A. (1973), *CRC Crit. Rev. Microbiol. 2,* 553.

Gill, D. M. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2064.

Gill, D. M., and King, C. A. (1975), *J. Biol. Chem. 250,* 6424.

Holmgren, J., Lonnroth, I., and Svennerholm, L. (1973a), *Infect. Immun. 8,* 208.

Holmgren, J., Lonnroth, I., and Svennerholm, L. (1973b), *J. Infect. Dis. 5,* 77.

King, C. A., and Van Heyningen, W. E. (1973), *J. Infect. Dis. 127,* 639.

Lai, C. Y., Mandy, E., and Chang, D. (1975), *J. Infect. Dis.* (in press).

Lo Spalluto, J. J., and Finkelstein, R. A. (1972), *Biochim. Biophys. Acta 257,* 158.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Pierce, N. F. (1973), *J. Exp. Med. 137,* 1009.

Rosen, O. M., and Erlichman, J. (1975), *J. Biol. Chem. 250,* 7788.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972a), *J. Biol. Chem. 247,* 36.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1972b), *J. Biol. Chem. 247,* 6135.

Rubin, C. S., Erlichman, J., and Rosen, O. M. (1974), *Methods Enzymol. 38,* 308.

Rubin, C. S., and Rosen, O. M. (1973), *Biochem. Biophys. Res. Commun. 50,* 421.

Van Heyningen, S. (1974), *Science 183,* 656.

Van Heyningen, S., and King, C. A. (1975), *Biochem. J.* 146, 269.

Van Heyningen, W. E., Carpenter, C. C. J., Pierce, N. F., and Greenough, W. B. III (1971), *J. Infect. Dis. 124,* 415.