Relationship of Phosphorylation and ADP-ribosylation Using a Synthetic Peptide as a Model Substrate*

Shanta V. Kharadia and Donald J. Graves‡

From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50011

Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly) is a good substrate for cholera toxin in comparison with the angiotensin peptides. Because kemptide contains two potential ADP-ribosylation sites and, is also a good substrate for cAMP-dependent protein kinase, it was possible to gain some insight into factors influencing the specificity of cholera toxin and to study the relationship between phosphorylation and ADP-ribosylation. The ADP-ribosylated products of kemptide were purified by high-performance liquid chromatography and characterized by peptide sequence analysis, trypsin digestion, and fast-atom bombardment mass spectrometry. The major product is mono(ADP-ribosyl)ated preferentially on the first arginyl residue and some mono(ADP-ribosyl)ation was observed to occur on the second arginine. The minor product is di(ADP-ribosyl)ated. The $K_m$ and $V_{max}$ for mono(ADP-ribosyl)ation of kemptide are approximately $4.3 \pm 1.2$ mm and $38.1 \pm 5.5$ mol min$^{-1}$ mg$^{-1}$, respectively. Phosphorylated seryl residue of kemptide suppresses ADP-ribosylation of the arginyl residues by cholera toxin. Mono(ADP-ribosyl)ated kemptide is a poor substrate for the cAMP-dependent protein kinase in comparison with kemptide. Di(ADP-ribosyl)ated kemptide is not phosphorylated at all. These results suggest that a mere exposure of an arginyl residue in peptides is not a sufficient condition for effective ADP-ribosylation and that a relationship exists between ADP-ribosylation and phosphorylation.

Cholera toxin catalyzes the transfer of the ADP-ribose moiety from NAD$^+$ to a variety of arginine-containing proteins and is also known to catalyze the hydrolysis of NAD$^+$ to ADP-ribose and nicotinamide (1). Among the most widely known proteins ADP-ribosylated by cholera toxin are the guanine nucleotide-binding regulatory proteins that inhibit the GTPase activity and concomitantly activate adenylate cyclase activity (2-4). Cholera toxin also ADP-ribosylates a variety of low molecular weight guanidine-containing compounds (5-7) and guanylylhydrazones (8). Many other proteins ADP-ribosylated by cholera toxin have been identified (9-13), but there is little knowledge of the specificity determinants of cholera toxin.

ADP-ribosyltransferases are present in many animal tissues (14-19), and the presence of enzymes that hydrolyze ADP-ribose attached to proteins via arginine linkage has been detected (20-22). Recent studies indicate that ADP-ribosylation may play a vital role in regulation of certain metabolic processes. In Rhodospirillum rubrum, a free-living, purple photosynthetic bacterium, the nitrogenase activity is regulated by reversible ADP-ribosylation of a specific arginyl residue of dinitrogenase reductase (23, 24). Also, several lines of evidence have accumulated that suggest a possible regulation of protein phosphorylation by ADP-ribosylation. Tani-gawa et al. (25, 26) have reported that ADP-ribosylation of histone and phosphorylase kinase (27) by hen liver nuclei ADP-ribosyltransferase suppresses subsequent phosphorylation by cAMP-dependent protein kinase.

Our laboratory has reported the presence of an endogenous ADP-ribosyltransferase in rabbit skeletal muscle (16), and this enzyme has been shown to share many properties with cholera toxin (28). Little is known about the specificity determinants for these ADP-ribosylating enzymes. Kemptide (Leu-Arg-Ala-Ser-Leu-Gly), an excellent substrate for cAMP-dependent protein kinase (29), has also been reported to be a good substrate for cholera toxin (30). We have found that kemptide is a better substrate for cholera toxin than the angiotensin peptides. Cholera toxin preferentially catalyzes ADP-ribosylation of the first arginyl residue of kemptide and that formation of di(ADP-ribosyl)ated kemptide also occurs. Using kemptide as a model substrate, we found that ADP-ribosylation suppresses phosphorylation by cAMP-dependent protein kinase and that phosphorylation decreases the rate of ADP-ribosylation by cholera toxin.

MATERIALS AND METHODS

Dithioerythritol, dithiothreitol, &-NAD', ADP-ribose, angiotensin peptides (Ser-Arg-Val-Tyr-Ile-His-Pro-Ala, Arg-Val-Tyr-Ile-His-Pro-Phe, Asp-Arg-Val-Tyr-Ile-His-Pro-Phe, and Asn-Arg-Val-Tyr-Val-His-Pro-Phe), kemptide (Leu-Arg-Ala-Ser-Leu-Gly), chelation (catalytic A subunit), and cAMP-dependent protein kinase (catalytic subunit) were obtained from Sigma. All other reagents used were of analytical grade.

High-performance liquid chromatography was performed on a Beckman 532M liquid chromatograph with a Beckman 155 Variable Detector interfaced with a Nelson Analytical 3500 Data System. A VYDAC 218TP C-18 reverse-phase column (4.6 x 150 mm) was used for all assays, and a larger column (10 x 250 mm) of the same type was used for purification of the products. Elution was accomplished with 0.1% trifluoroacetic acid (buffer A) and 0.08% trifluoroacetic acid in 100% acetonitrile (buffer B) at a flow rate of 1.0 ml/min. A linear gradient from 0 to 24% buffer B in 25 min and then to 100% buffer B in 4 min was used. With the larger column, the flow rate used was 3.0 ml/min.

Negative-ion fast-atom bombardment mass spectrometry was performed on a Kratos MS-50TC Mass Spectrometer. Peptide sequencing was performed on an Applied Biosystems model 470A Protein Sequencer with an on-line model 120A phenylthiocarbamoyl amino acid analyzer. Amino acid analysis was performed on an Applied Biosystems 150A amino acid analyzer coupled with an Applied Bio-

Received for publication, June 1, 1987)
systems 430A derivatizer, and a Beckman DU-7 Spectrophotometer was used for all spectrophotometric measurements.

**ADP-ribosylation of Peptides**—An assay mixture consisting of 2 mM peptide arginy1 residue, 10 mM NAD, 20 mM diethiothreitol, 50 µg/ml cholera toxin A subunit, and 100 mM glycylglycine ethyl ester buffer (pH 7.6) in a total volume of 25 µl was incubated at 30 °C. Mixture A (consisting of glycylglycine ethyl ester, diethiothreitol, and cholera toxin) and mixture B (consisting of NAD, peptide, and H2O) were preincubated at 30 °C for 20 min to avoid an initial lag in the reaction. The reaction was initiated by addition of mixture A to mixture B. Aliquots (10 µl) were removed from the assay system at 0 and 30 min and added to 10 µl of 10% trichloroacetic acid to stop the reaction. The samples were diluted to 300 µl, centrifuged, and injected into HPLC. The peaks from the chromatogram at 260 nm were integrated.

**Purification of ADP-ribosylated Products of Kemptide**—The reaction system was set up as described with 2 mM kemptide in a total volume of 250 µl. After incubation for 6-8 h at 30 °C, the reaction was terminated with an equivalent volume of 10% trichloroacetic acid. The contents were filtered and injected into HPLC and a larger VYDAC column was used. The chromatography was monitored at 260 nm, and the product peaks were collected and lyophilized. The lyophilized products were reconstituted with water and were again purified by HPLC and lyophilized. The products were then reconstituted with water, and the concentrations were determined by spectrophotometric measurement of their absorbance at 260 nm and by using the extinction coefficient for ADP-ribose (15,640 M⁻¹ cm⁻¹), also determined spectrophotometrically. A standard curve for mono(ADP-ribosyl)ated kemptide was constructed by using HPLC.

**Acetylation of Kemptide**—Kemptide (0.62 mg) was dissolved in 500 µl of saturated solution of sodium acetate (pH 5.5) followed by gradual addition of acetic anhydride for 1 h (acetylation was carried out on ice). The resulting mixture was stirred for 1 h. The two products formed were purified by reverse-phase HPLC.

**Trypsin Digestion**—The reaction system consisted of 10 mM CaCl₂, 100 µg/ml trypsin, 1.84 mM substrate (ADP-ribosylated products), and 50 mM ammonium bicarbonate (pH 7.76) in a total volume of 100 µl. After approximately 4 h of incubation at 37 °C, the reaction was terminated by the addition of 5 µl of 100% trichloroacetic acid. The contents were filtered and assayed by HPLC, and the chromatograms were monitored at 214 and 260 nm.

**Purification of Phosphorylated Kemptide**—The reaction system consisted of 10 mM ATP, 62.5 mM MES (pH 6.5), 12.5 mM MgCl₂, 0.25 mM EGTA, 10 µg/ml of the catalytic subunit of cAMP-dependent protein kinase, and 5 mM kemptide in a total volume of 500 µl. After approximately 5 h of incubation at 30 °C, the reaction was terminated with an equivalent volume of 10% trichloroacetic acid. The sample was filtered, injected into HPLC, and purified by using the larger VYDAC column. Subsequent purification steps were identical to those for purification of ADP-ribosylated products. The concentration of phosphorylated kemptide was determined by amino acid analysis.

## Results

**ADP-ribosylation of Arginine-containing Peptides**—The results of Table I show that angiotensin peptides and kemptide are substrates for cholera toxin and that not all arginyl residues are modified equally. The two octapeptides, 3 and 4, essentially differ in the N-terminal amino acid, but peptide 4 with an asparaginyl residue seems to be a much better substrate than peptide 3 with an aspartyl residue. Peptide 2, missing an amino acid at the N terminus, is as good a substrate as peptide 3. Peptide 1, an octapeptide with sarcosine (N-methyl glycine) at the N terminus, seems to be a better substrate than peptides 2, 3, and 4. These preliminary results seem to indicate that a hydrophobic amino acid residue near an arginyl residue results in increased amount of ADP-ribosylation. Kemptide, a heptapeptide containing two arginyl residues, is the best substrate for cholera toxin in comparison with the angiotensin peptides. ADP-ribosylation of kemptide was investigated further because it is a good substrate for cholera toxin with two potential sites for ADP-ribosylation and has been shown to be an excellent substrate for cAMP-dependent protein kinase, making it possible to study the relationship between phosphorylation and ADP-ribosylation.

**ADP-ribosylation of Kemptide**—Incubation of kemptide with NAD⁺ and cholera toxin resulted in the formation of two products, which were separated by reverse-phase HPLC (Fig. 1A). Both minor (peak a) and major (peak b) product peaks absorb at 260 and 214 nm. Kemptide absorbs only at 214 nm, and the absorption of the products at 260 nm is most likely due to the ADP-ribose moiety. The two product peaks were purified by HPLC.

To characterize the nature of the products, both minor and

---

**Table I**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>ADP-ribosylated product* %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Sar-Arg-Val-Tyr-Ile-His-Pro-Ala</td>
<td>82</td>
</tr>
<tr>
<td>2 Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>27</td>
</tr>
<tr>
<td>3 Asp-Arg-Val-Tyr-Ile-His-Pro-Phe</td>
<td>25</td>
</tr>
<tr>
<td>4 Asn-Arg-Val-Tyr-Val-His-Pro-Phe</td>
<td>58</td>
</tr>
<tr>
<td>2 Leu-Arg-Arg-Ala-Ser-Leu-Gly</td>
<td>100</td>
</tr>
</tbody>
</table>

* The numbers represent percentage areas obtained by integration of the product peak at 260 nm and the absorbance at 260 nm is primarily due to ADP-ribose. The percentages are computed by making kemptide 100% (approximately 1.1 nmol of mono(ADP-ribosyl)ated kemptide was formed).

---

**Fig. 1.** A, cholera-toxin catalyzed ADP-ribosylation of kemptide. The HPLC profile shows the formation of two products; a minor (a) and a major (b) product. The reaction was incubated for 3-4 h at 30 °C. Other conditions are as described under "Materials and Methods." B, HPLC analysis of tryptic digestion products of mono(ADP-ribosyl)ated kemptide. The reaction system was set up as described under "Materials and Methods." The tryptic digests are indicated by numbers. The peak eluting at approximately 18 min is a contaminant present in the reaction mixture and the peak eluting at 23.5 min is undigested mono(ADP-ribosyl)ated kemptide.
major products and kemptide were sequenced (Table II). The yields of the arginyl residues and several other residues in kemptide are in the same range. The yields of the two arginyl residues in the major product, in contrast, differ by approximately 71%. The lower yield of the first arginyl residue of the major product may suggest that it is modified producing mono(ADP-ribosyl)ated kemptide. In the minor product, however, the yields of the two arginyl residues are equally low, possibly suggesting modification of both of the arginyl residues. The elution pattern of the products, the absorption at 260 nm due to the ADP-ribose moiety, and peptide sequence analysis suggest that the major product may be mono(ADP-ribosyl)ated preferentially at the first arginyl residue, and the minor product, di(ADP-ribosyl)ated. Negative-ion FAB mass spectrometry of the major product showed an unprotonated molecular ion at m/z 1312, corresponding to the molecular weight of the mono(ADP-ribosyl)ated kemptide (M, = 1313).

The peptides were subjected to trypsin digestion to further define the sites of modification. Treatment of the minor product with trypsin did not produce any fragments, suggesting that both arginyl residues are ADP-ribosylated and, as a result, are resistant to cleavage by trypsin. Tryptic cleavage of the major product, in contrast, produced five peptide fragments (Fig. 1B). Fragments 2, 4, and 5 also absorb at 260 nm. Absorbance at 260 nm is due to the ADP-ribose moiety of the fragment. Fragment 2 constitutes approximately 70% of the total product absorbing at 260 nm, and fragments 4 and 5 constitute the remaining 30%. Fragments 2, 4, and 5 were purified by reverse-phase HPLC and subjected to negative-ion FAB mass spectrometry. Mass spectrum of fragment 2 showed an unprotonated molecular ion at m/z 983, corresponding to the molecular weight of the ADP-ribosylated fragment, Leu-Arg(ADP-ribose)-Arg (M, = 984). This fragment can only result from the cleavage after the second arginyl residue of the mono(ADP-ribosyl)ated kemptide, indicating that the first arginyl residue of this fragment must be modified and not the second because it was shown, using di(ADP-ribosyl)ated kemptide, that trypsin will not cleave after an ADP-ribosylated arginine. Hence, approximately 70% of the total mono(ADP-ribosyl)ated product contains the ADP-ribose attached to the first arginyl residue.

Negative-ion FAB mass spectrometry of fragment 5 showed an unprotonated molecular ion at m/z 1042, corresponding to the molecular weight of the ADP-ribosylated pentapeptide, Arg(ADP-ribose)-Ala-Ser-Leu-Gly (M, = 1043). A mass spectrum of fragment 4 could not be obtained, but it must also contain an ADP-ribose moiety because it absorbs at 260 nm. Both of these fragments were shown to have identical sequence, X-Ala-Ser-Leu-Gly, as determined by peptide sequencing. The first cycle of Edman degradation did not show presence of any amino acid. Because the yield of the modified arginyl residues was low for the mono- and di(ADP-ribosyl)ated kemptide (Table II), it is highly possible that the first residue, X, in the pentapeptide is ADP-ribosylated arginine and, as a result, is not detected. Some breakdown of ADP-ribosyl arginine may have occurred and is not detected because of small quantity of the sample used in the analysis. These results indicate that fragments 4 and 5 are the same material, Arg(ADP-ribose)-Ala-Ser-Leu-Gly, resulting from mono(ADP-ribosylation of the second arginyl residue of kemptide. Hence, approximately 30% of the mono(ADP-ribosyl)ation may be occurring at the second arginyl residue. Fragment 1 was shown to co-elute with the dipeptide, Leu-Arg and fragment 3 is likely Ala-Ser-Leu-Gly, resulting from cleavage after the second arginyl residue. Peptide sequence analysis, trypsin digestion, and FAB mass spectrometry show that mono(ADP-ribosyl)ation occurs preferentially on the first arginyl residue and that the minor product is di(ADP-ribosyl)ated.

### Table II

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Major product</th>
<th>Minor product</th>
<th>Kemptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu</td>
<td>1417</td>
<td>590</td>
<td>1709</td>
</tr>
<tr>
<td>Arg</td>
<td>128</td>
<td>40</td>
<td>136</td>
</tr>
<tr>
<td>Arg</td>
<td>440</td>
<td>39</td>
<td>147</td>
</tr>
<tr>
<td>Ala</td>
<td>1118</td>
<td>406</td>
<td>1235</td>
</tr>
<tr>
<td>Ser</td>
<td>537</td>
<td>186</td>
<td>117</td>
</tr>
<tr>
<td>Leu</td>
<td>654</td>
<td>146</td>
<td>817</td>
</tr>
<tr>
<td>Gly</td>
<td>270</td>
<td>98</td>
<td>130</td>
</tr>
</tbody>
</table>

**Determination of the sequence of ADP-ribosylated products**

### Enzymatic and Nonenzymatic Mono(ADP-ribosyl)ation of Kemptide—To evaluate the effectiveness of kemptide as a substrate for ADP-ribosylation, kinetic studies were undertaken, but we were unable to obtain a saturation curve for kemptide in presence of 100 mM phosphate buffer (pH 7.5). The formation of the product was linear with respect to substrate concentration. A possible occurrence of a nonenzymatic mono(ADP-ribosyl)ation of kemptide in the reaction system could explain this phenomenon. Upon incubation of 5 mM kemptide with 10 mM ADP-ribose, 20 mM DTE, and 100 mM phosphate buffer (pH 7.5) at 30 °C for 1 h, HPLC revealed a product peak that absorbed at both 214 and 260 nm and co-eluted with the enzymatic mono(ADP-ribosyl)ated kemptide (results not shown). Hence, the linear increase in velocity with increasing kemptide concentration may be due to a similar type of chemical reaction occurring in the reaction system in as much as cholera toxin can catalyze the hydrolysis of NAD⁺ to ADP-ribose and nicotinamide, providing the free ADP-ribose for the chemical reaction. No such chemical reaction was observed when kemptide was incubated with 10 mM NAD⁺, 20 mM DTE, and 100 mM phosphate buffer (pH 7.5).

To identify the site of chemical modification, kemptide was acetylated with acetic anhydride to block the α-amino group of kemptide. Incubation of the acetylated kemptide products with 10 mM ADP-ribose, 20 mM DTE, and 100 mM phosphate buffer (pH 7.6) did not result in the production of a chemical product. Enzymatic products were formed when the acetylated kemptide was incubated with cholera toxin and NAD⁺. These results suggest that ADP-ribose reacts with the α-amino group of the peptide and does not chemically react with the guanido side-chain group. The nonenzymatic product from the reaction of kemptide with ADP-ribose was purified by reverse-phase HPLC. The enzymatic and nonenzymatic products were differentiated by their sensitivity to 1.0 M hydroxylamine at pH 7.5 at 37 °C. The enzymatic product was cleaved by hydroxylamine, producing free kemptide and a product that co-eluted with ADP-ribose. The nonenzymatic mono(ADP-ribosyl)ated kemptide, in contrast, was not affected by hydroxylamine under the same conditions.

Because of the nonenzymatic reaction of ADP-ribose with the α-amino group of kemptide, high concentrations of glycine ethyl ester (100 mM) were included in the reaction system to act as a scavenger of any free ADP-ribose generated during the reaction. Effectiveness of glycine ethyl ester was demonstrated by incubation of kemptide with 10 mM ADP-ribose, 20 mM DTE, and 100 mM glycine ethyl ester (pH 7.6). Reduction in the formation of the nonenzymatic product was observed in this system. In the presence of glycine ethyl ester, we were able to obtain a saturation curve for kemptide (Fig.
Effect of ADP-ribosylation on phosphorylation—Mono- and di(ADP-ribosyl)ated products of kemptide were used as substrates for cAMP-dependent protein kinase to evaluate the effect of ADP-ribosylation on phosphorylation (Fig. 3). The initial velocity for phosphorylation of kemptide is approximately 15-fold greater than the initial velocity for phosphorylation of the mono(ADP-ribosyl)ated kemptide. The di(ADP-ribosyl)ated kemptide is not phosphorylated at all. These results show that ADP-ribosylation suppresses phosphorylation.

Effect of Phosphorylation on ADP-ribosylation—To determine whether phosphorylation of the seryl residue of kemptide could affect ADP-ribosylation, kemptide was phosphorylated with cAMP-dependent protein kinase, purified, and subjected to ADP-ribosylation by cholera toxin (Fig. 4). Phosphorylated kemptide ($M_r = 852$) was characterized by negative-ion FAB mass spectrometry which showed a molecular ion at $m/z$ 851. Phosphorylated kemptide was not ADP-ribosylated during approximately $4.5$ h of incubation with cholera toxin. With prolonged incubation, however, a small amount of phosphorylated kemptide was ADP-ribosylated. With high activity of cholera toxin, ADP-ribosylation of phosphokemptide was observed at lower time points, but the extent of ADP-ribosylation was still considerably less (approximately 2.4-fold) as compared with ADP-ribosylation of kemptide. The phosphate group on the seryl residue of kemptide causes a decrease in the rate of ADP-ribosylation by cholera toxin.

**DISCUSSION**

The use of a well-defined substrate, Leu-Arg-Arg-Ala-Ser-Leu-Gly, was important in this study because it allowed us to perform preliminary studies on the specificity requirements of ADP-ribosylation and to examine the possible relationship of ADP-ribosylation and phosphorylation. Cholera toxin was used as a model enzyme for guanidine-specific mono(ADP-ribosyl)transferases because it is well defined (31–33) and has similar enzymatic activity to other ADP-ribosyltransferases with small molecular weight guanidine-containing compounds (8, 34). This study of cholera toxin with peptide substrates shows that mere exposure of an arginyl side chain is not a sufficient condition for effective ADP-ribosylation.

Three products, two mono(ADP-ribosyl)ated derivatives and a di(ADP-ribosyl)ated peptide, have been identified for reaction of cholera toxin with kemptide. Peptide sequence analysis, trypsin digestion, and FAB mass spectrometry show that approximately 70% of the major mono(ADP-ribosyl)ated product is due to a reaction on the first arginyl residue. Modification has also been proved to occur on the second arginyl residue. The di-product is formed, showing that an ADP-ribosyl group on an adjacent arginyl residue will not block further reaction. Further information on factors influencing ADP-ribosylation was obtained by using a phosphorylated peptide. In this case, little ADP-ribosylation occurred. It is possible, however, that the negative charge on the phosphoryl group may weaken the interaction between the enzyme and the substrate. The negatively charged aspartyl residue of peptide 3 (Table I) also seems to reduce ADP-ribosylation.
These results show that ADP-ribosylation occurs preferentially on the first arginyl residue, di(ADP-ribosyl)ation can occur, and that a negatively charged group near an arginyl residue suppresses ADP-ribosylation.

Our studies with kemptide also point out that free ADP-ribose generated from the hydrolysis of NAD$^+$ by choleratoxin can chemically react with kemptide, producing a product that co-elutes with the enzymatic product on HPLC, but differs in that it is stable to hydroxylamine. No saturation kinetics could be obtained unless glycine ethyl ester is used in the reaction mixture. Our results suggest that the chemical reaction occurs at the α-amino group of the peptide and that the amino group of the buffer, glycine ethyl ester, counteracts this reaction. Hilt et al. (35) have demonstrated earlier that free ADP-ribose generated by NAD glycohydrolase activity can react nonenzymatically to give a product that is hydroxylamine resistant, but they did not identify the acceptor site. Hence, further kinetic studies should be done under conditions that avoid the chemical reaction. ADP-ribosylation of angiotensin peptides in glycine ethyl ester buffer (Table I) show that the extent of the reaction of these peptides was less in comparison with kemptide, further indicating that the environment around the reacting arginyl residue influences the effectiveness of ADP-ribosylation. Kinetic studies are in progress to ascertain more completely the significance of these differences.

The studies of Tanigawa et al. (25, 27) on protein substrates, histones, and phosphorylase kinase suggest strongly that ADP-ribosylation affects protein phosphorylation. We found similar results with our model peptide substrates, the mono(ADP-ribosyl)ated product being phosphorylated more poorly than kemptide and the di(ADP-ribosyl)ated product not at all. Cyclic AMP-dependent protein kinase clearly has a requirement for arginyl residues on the amino-terminal side of the phosphorylatable serine, and ADP-ribosylation could prevent access of the modified peptides to the active site region. Tanigawa et al. (25) found no effect of phosphorylation on ADP-ribosylation of histones. This is in contrast to our results with a peptide substrate. Because histones contain many arginyl residues and, hence, many potential sites for ADP-ribosylation, the lack of an effect of phosphorylation on ADP-ribosylation is not surprising. The study with a peptide substrate shows clearly that phosphorylation affects ADP-ribosylation in the immediate environment and suggests that effects of phosphorylation in proteins might be constrained to nearby arginyl residues. Our current work and results of Tanigawa et al. suggest a possible relationship between phosphorylation and ADP-ribosylation. This question is being investigated further in our laboratory.

Acknowledgments—We thank Dr. Louise Tabatabai of the National Animal Disease Center, Ames, Iowa, for performing the protein sequencing, Shirley Sayre for performing the amino acid analyses, Toni Caputo for her initial studies, and Dr. Gopalan Soman for his suggestions regarding this work.

REFERENCES