Bruton’s Tyrosine Kinase Is Involved in p65-mediated Transactivation and Phosphorylation of p65 on Serine 536 during NFκB Activation by Lipopolysaccharide*

Sarah L. Doyle, Caroline A. Jefferies‡, and Luke A. O’Neill§§

From the Department of Biochemistry and Biotechnology Institute, Trinity College, Dublin 2, Ireland

Bruton’s tyrosine kinase (Btk) has recently been shown to participate in the induction of nuclear factor κB (NFκB)-dependent gene expression by the lipopolysaccharide (LPS) receptor Toll-like receptor-4 (TLR4). In this study we have examined the mechanism whereby Btk participates in this response. Treatment of the murine monocytic cell line Raw264.7 with LFM-A13, a specific Btk inhibitor, blocked LPS-induced NFκB-dependent reporter gene expression but not IκB degradation. Transient transfection of HEK293 cells with Btk had no effect on NFκB-dependent reporter gene expression but strongly promoted transactivation of a reporter gene by a p65-Gal4 fusion protein. IκB degradation activated by LPS was intact in macrophages from X-linked immunodeficiency (Xid) mice, which contain inactive Btk. Transfection of cells with a dominant negative form of Btk (BtkK430R) inhibited LPS-driven p65 mediated transactivation. Additionally LFM-A13 impaired phosphorylation of serine 536 on p65 induced by LPS in HEK293-TLR4 cells, and in Xid macrophages this response was impaired. This study therefore reveals a novel function for Btk. It is required for the signaling pathway activated by TLR4, which culminates in phosphorylation of p65 on serine 536 promoting transactivation by NFκB.

Bruton’s tyrosine kinase (Btk) is a member of the Tec family of non-receptor tyrosine kinases and is found in all cells of the hematopoietic lineage except plasma cells and T-cells (1). In B-cells, activation of the B-cell receptor leads to Btk being rapidly recruited to the plasma membrane where it becomes phosphorylated and activated. Btk is required for normal B-cell development (2) and the gene encoding Btk was first identified as the mutated gene in X-linked agammaglobulinemia (XLA) in humans, an immune disease characterized by a lack of circulating B lymphocytes and an absence of Igs of all classes (3). Mutations in the Btk gene also result in the less severe X-linked immunodeficiency (Xid) in mice. Various mutations have been identified as being responsible for the XLA phenotype, whereas the mutation in Xid mice has been mapped to arginine 28 (R28C) in the PH domain (4). This prevents Btk associating with the membrane, thus inactivating it. The PH domain is important in the process of Btk activation, since it localizes Btk to the membrane through its interaction with phosphatidylinositol 3-kinase (PI3K).

Due to the major phenotype of Btk deficiency being impaired B-cell development and function, the main focus of interest in Btk has centered around the B-cell. However, several studies have provided a more general role for Btk in immune regulation. Studies in Xid peritoneal macrophages have shown reduced responses to lipopolysaccharide (LPS) stimulation, with tumor necrosis factor α (TNFα) and IL-β production decreased and macrophage effector functions impaired (5). We have shown that Btk is recruited to the LPS receptor Toll-like receptor-4 (TLR4) where it is activated and is required for NFκB activation (6). In addition, peripheral blood mononuclear cells (PBMCs) isolated from XLA patients have been shown to have impaired TNFα production induced by LPS (7).

The mechanism whereby Btk participates in TLR4 signaling to NFκB is not known. The prototypical form of NFκB is a heterodimer consisting of a 50-kDa DNA-binding subunit (p50) and a 65-kDa transactivation subunit (RelA/p65). NFκB is ubiquitously expressed and in uninduced cells is retained in the cytoplasm in an inactive form by the IκB family of proteins. Upon stimulation of cells with LPS four adapter proteins are engaged, MyD88, MyD88 adapter-like (Mal), Toll/IL-1 receptor domain-containing adapter-inducing interferon β (TRIF), and TRIF-related adapter molecule (reviewed in Ref. 8). MyD88 and Mal engage with IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1 leading to activation of the IκB kinase (IKK) complex via Traf-6 (9, 10). In this pathway IκBα becomes phosphorylated by the IKK complex and is subsequently targeted for ubiquitination and degradation (11, 12). This results in the release of NFκB, which rapidly translocates into the nucleus, binds to its target DNA triggering the transcription of various target genes (13). In addition, LPS signaling promotes phosphorylation of the p65 subunit of the NFκB complex, which is required for transactivation of gene expression. To date, five distinct serine residues have been identified on p65 that are inducibly phosphorylated in response to TNFα, IL-1, and/or LPS. Serine 276 on p65 is phosphorylated by protein kinase A. LPS induces serine 536 phosphorylation, which may involve IKK2 (14). Other candidate kinases include IKKe and TBK1, and an

Received for publication, February 8, 2005, and in revised form, April 15, 2005
Published, JBC Papers in Press, April 22, 2005, DOI 10.1074/jbc.C500053200

* This work was supported by Science Foundation Ireland. 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.
‡ These authors contributed equally to this study.
§ To whom correspondence should be addressed. Tel.: 353-1-608-2439; Fax: 353-1-677-2400; E-mail: laoneil@tcd.ie.

1 The abbreviations used are: Btk, Bruton’s tyrosine kinase; NFκB, nuclear factor κB; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; IκBα, inhibitory protein κB; HEK, human embryonic kidney; XLA, X-linked agammaglobulinemia; Xid, X-linked immunodeficiency; PH, pleckstrin homology; TNF, tumor necrosis factor; IL, interleukin; PBMC, peripheral blood mononuclear cells; Mal, MyD88 adapter-like; TRIF, Toll/IL-1 receptor domain containing adapter-inducing interferon β (TRIF), and TRIF-related adapter molecule (reviewed in Ref. 8). MyD88 and Mal engage with IL-1 receptor-associated kinase (IRAK)-4 and IRAK-1 leading to activation of the IκB kinase (IKK) complex via Traf-6 (9, 10). In this pathway IκBα becomes phosphorylated by the IKK complex and is subsequently targeted for ubiquitination and degradation (11, 12). This results in the release of NFκB, which rapidly translocates into the nucleus, binds to its target DNA triggering the transcription of various target genes (13). In addition, LPS signaling promotes phosphorylation of the p65 subunit of the NFκB complex, which is required for transactivation of gene expression. To date, five distinct serine residues have been identified on p65 that are inducibly phosphorylated in response to TNFα, IL-1, and/or LPS. Serine 276 on p65 is phosphorylated by protein kinase A. LPS induces serine 536 phosphorylation, which may involve IKK2 (14). Other candidate kinases include IKKe and TBK1, and an
as yet unknown kinase identified by chromatic fractionation of cell extracts (15) prepared from cells treated with IL-1, which signals in a similar manner to LPS (16). The details of the pathway activated by TLR4 leading to phosphorylation of p65 on serine 536 are, however, not fully understood.

Having previously shown that LPS activated Btk and that inactive mutants of Btk inhibited LPS signaling to NFκB-linked gene expression, we have investigated the function of Btk in NFκB regulation. In the present study we have found that Btk is involved in the pathway leading to phosphorylation of p65 on serine 536 and is not involved in the process leading to IκBα degradation. Our study therefore elucidates the role of Btk in TLR4 signaling and implicates Btk for the first time in the transactivation pathway of NFκB.

MATERIALS AND METHODS

Cell Culture—HEK293, U373, and HEK293-TLR4 cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 100 μg/ml gentamicin and 2 mM L-glutamine. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2.

Plasmids and Reagents—The plasmid encoding the chimeric CD4-CD4-TLR4 was a kind gift from Dr. R. Medzhитов (Yale University, New Haven, CT) and has been described previously (17). The NFκB-luciferase plasmid was a kind gift from Dr. R. Hofmeister (Universitat Regensburg, Regensburg, Germany) and contains 5κB sites upstream of the luciferase gene. The plasmid encoding wild-type Btk was obtained from R. Hendriks (Rotterdam, the Netherlands), and the dominant negative Btk (K430R) plasmid has been described previously (6). Galα-p65/S536A plasmid encoding p65 subunit of NFκB fused to the Galα DNA-binding domain was a kind gift from Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and has been described elsewhere (18). The Galα-luciferase reporter gene was obtained from Stratagene. The monoclonal p65 antibody was obtained from Santa Cruz Biotechnology, Inc., the polyclonal phospho-NFκB plasmid, Galα-p65/S536A plasmid encoding p65 subunit of NFκB fused to the Galα DNA-binding domain was a kind gift from Lienhard Schmitz (German Cancer Research Center, Heidelberg, Germany) and has been described elsewhere (18). The Galα-luciferase reporter gene was obtained from Stratagene. The monoclonal p65 antibody was obtained from Santa Cruz Biotechnology, Inc., the polyclonal phospho-NFκB (Ser536) antibody was obtained from Cell Signaling Technology Inc., the monoclonal anti-β-actin antibody was obtained from Sigma (Poole, UK), and the IκBα antibody was a kind gift from Prof. R. Hay (University of St. Andrews, St. Andrews, UK). The Btk-specific inhibitor, LFM-A13, was obtained from C. Haven (Trinity College, Dublin, Ireland). Peritoneal macrophages were prepared from R. Hendriks (Rotterdam, the Netherlands), and the dominant negative Btk (K430R) and the Btk-selective kinase inhibitor LFM-A13, on the induction of an NFκB-linked reporter gene comprising five NFκB sites linked to luciferase. We had previously shown that Btk/K430R could block CD4-TLR4-driven NFκB-linked luciferase in 293 cells (6); we now confirm that finding in the LPS-responsive, murine monocytic cell line RAW264.7. The ability of LPS to drive NFκB-linked luciferase was abolished by transfecting cells with a plasmid encoding Btk/K430R (Fig. 1a). We also tested the effect of the Btk inhibitor LFM-A13 on LPS-induced NFκB-activation. Pretreatment of RAW264.7 cells with 100 μM LFM-A13 reduced LPS-induced NFκB activation by 70% (Fig. 1b). We next examined the activation of IκBα degradation using the LPS-responsive cell line HEK293-TLR4. As shown in Fig. 1c, LPS induced IκBα degradation in these cells from 30 min stimulation (left-hand panel). Pretreating the cells with LFM-A13 had no effect on this degradation (right-hand panel). To further test the possible role of Btk in LPS-induced IκBα degradation, we isolated bone marrow-derived macrophages (BMDMs) and peritoneal macrophages from Xid and normal mice. Macrophages from Xid mice lack a functioning Btk (4). We confirmed the phenotype of the Xid mice, demonstrating a lack of Btk activation in response to LPS (not shown). As shown in Fig. 1d LPS-induced IκBα degradation was identical in both normal and Xid BMDMs, with almost complete degradation occurring from 15 min stimulation (Fig. 1d, lane 3) in both cases, indicating no obvious impairment of the IκBα degradation pathway. LPS caused a classical response in the degradation of IκBα in both wild-type and Xid BMDMs, with a doublet appearing after 3 min stimulation indicative of phosphorylation, degradation occurring from 15 min, and IκBα induction occurring from 45 min. Similarly there was no impairment of IκBα degradation in Xid peritoneal macrophages when compared with normal peritoneal macrophages, with complete degradation occurring from 15 min LPS stimulation (Fig. 1e, lane 3).

Btk Is Involved in LPS-induced p65-mediated Transactivation—The ability of dominant negative Btk and LFM-A13 to inhibit NFκB-linked luciferase expression and yet the lack of a role for Btk in the degradation of IκBα in response to LPS pointed toward a role for Btk on the p65-mediated transcription pathway of NFκB. To test this hypothesis we utilized the Galα-p65/S536A trans-reporting system described previously (19, 20). Briefly, this assay employs an expression plasmid encoding the transactivation p65 subunit of NFκB fused to the DNA-binding domain of Galα and a Galα-responsive reporter plasmid, Gal-luciferase. The advantage of this system is that
Gal4-p65$^{1-551}$ is regulated independently of IxBα, allowing the effect of LPS on the p65-transactivation pathway to be assessed. Transient transfection of HEK293 cells with increasing amounts of plasmid expressing wild-type Btk (Fig. 2a, left-hand panel) resulted in a dose-dependent increase in activation of Gal4-p65$^{1-551}$, with 100 ng of plasmid encoding Btk resulting in a 9-fold Gal4-p65$^{1-551}$ activation. However this Gal4-p65$^{1-551}$ activation was substantially reduced when Btk was cotransfected with a plasmid encoding Btk(K430R) (Fig. 2a). We also assessed the effect of Btk(K430R) on CD4-TLR4. Btk(K430R) abolished the effect of CD4-TLR4 on Gal4-p65$^{1-551}$ activity (Fig. 2e). We also investigated the effect of the Btk inhibitor LFM-A13 on LPS induced Gal4-p65$^{1-551}$ activation in HEK293-TLR4 cells. Pretreatment of the cells with 100 μM LFM-A13 reduced LPS-induced Gal4-p65$^{1-551}$ activation by 70% compared with control cells (Fig. 2f).

LPS-induced Phosphorylation of Serine 536 on p65 Is Inhibited by the Btk-specific Inhibitor LFM-A13 and Abolished in Cells from Xid Mice—Overall, the data suggested that there was a role for Btk in LPS-induced p65-mediated transactivation of NFκB. As Btk did not drive the mutant Gal4-p65$^{1-551}$ activity (Fig. 2e), we next investigated this response by employing a specific phospho-p65 antibody targeted against serine 536. LPS-induced phosphorylation of serine 536 increases p65 transcriptional activity (14), and as it has been reported that LPS induces the phosphorylation of NFκB in monocytes/macrophages, and furthermore, that the phosphorylation of serine 536 increases p65 transcriptional activity (14), we next investigated this response by employing a specific phospho-p65 antibody targeted against serine 536. LPS induced a strong increase in p65 phosphorylation on serine 536 in multiple cell types tested, including HEK293-TLR4 and U373 cells.
bruton's tyrosine kinase promotes p65-mediated transactivation. a, hek293 cells (2.5 x 10^6) were transfected with plasmids containing gal-luciferase (5 ng), renilla luciferase (40 ng), and plasmids expressing gal4-p65 (5 ng) (left-hand panel) or gal4-p65(536a) (right-hand panel) and btk or cd4-tlr4, as indicated. 18 h after transfection the cells were harvested, and luciferase activity was subsequently measured. results (mean ± s.d. for triplicate determinations) are represented as fold increase compared with unstimulated controls. b, hek293 cells (2.5 x 10^6) (left-hand panel) and hek293-tlr4 cells (2.5 x 10^6) (right-hand panel) were transfected with plasmids containing b-luciferase (80 ng) and renilla luciferase (40 ng) and a plasmid expressing wild-type btk as indicated or cd4-tlr4 (100 ng). 18 h after transfection the cells were either left untreated or treated with lps (1 μg/ml, 6 h) harvested, and luciferase activity was subsequently measured. results (mean ± s.d. for triplicate determinations) are represented as fold increase compared with unstimulated controls. c–f, hek293-tlr4 cells (2.5 x 10^6) were transfected with plasmids containing gal-luciferase (5 ng), renilla luciferase (40 ng), and a plasmid expressing gal4-p65 (5 ng). 18 h after transfection the cells were stimulated with lps at the indicated doses for 6 h or left untreated as shown (c), transfected for 18 h with a plasmid expressing btk(k430r) as indicated and treated with lps (1 μg/ml, 6 h) or tnfα (20 ng/ml, 3 h) (d), transfected with plasmids expressing cd4-tlr4 (50 ng) and btk(k430r) as indicated for 18 h (e), or pretreated with me2so (dms) as a control or the btk-specific inhibitor lfm-a13 as indicated for 45 min, after which cells were stimulated with lps (500 ng/ml, 6 h) as shown (f). results shown are mean ± s.d. for triplicate determinations and are either fold increase over untreated cells (a–c) or are represented as percentage of lps (d–f) or cd4-tlr4 stimulated cells (c). all results are representative of three separate experiments.

(fig. 3a, left- and right-hand panels, respectively). a response in both cell types from 15 min was evident. we examined the effect of lfm-a13 in hek293-tlr4 cells. as shown in fig. 3b p65 phosphorylation was greatly reduced when the cells were pretreated with lfm-a13; however, some residual phosphorylation was still evident (fig. 3b, compare lanes 6, 8, and 10 with lanes 5, 7, and 9, respectively).

we next tested the response in cells from wild-type and xid
mice. LPS caused a transient increase in p65 phosphorylation, which occurred at 15 min with the response decreasing at 45 min in wild-type peritoneal macrophages (Fig. 3c, left-hand panel). This response was much decreased in peritoneal macrophages from Xid mice (right-hand panel). In BMDMs, the increase in phosphorylation was also transient, occurring at 5 min and declining at 15 min (Fig. 3d, left-hand panel). This response was abolished in BMDMs from Xid mice (Fig. 3d, right-hand panel).

Taken together our results suggest a model whereby Btk is required for the TLR4-activated pathway leading to phosphorylation of p65 on serine 536, promoting transactivation of NFκB-dependent genes.

DISCUSSION

In this study we have found that Btk is involved in the LPS-induced pathway leading to phosphorylation of the p65 subunit of NFκB on serine 536, promoting transactivation of NFκB-dependent genes. We had previously discovered a role for Btk in TLR4 signaling to NFκB, demonstrating activation of Btk upon stimulation of cells with LPS, an interaction between Btk and TLR4, and an inhibitory effect of dominant negative Btk on NFκB-linked gene expression (6). Our study therefore pinpoints the process in the NFκB system which involves Btk, providing a novel function for the Btk enzyme.

Regulation of NFκB following stimulation with LPS occurs via activation of two pathways. The best characterized of these regulates the release of NFκB from its inhibitory protein IκB, subsequently allowing NFκB to translocate to the nucleus. However, this nuclear translocation of NFκB is not sufficient to activate NFκB-dependent gene transcription alone. The second pathway, which involves post-translational modifications, regulates the transactivating ability of the p65 subunit of NFκB once bound to its consensus sequence. Using the Btk-selective kinase inhibitor LFM-A13 and Xid macrophages, which lack a functioning Btk, our results clearly show no role for Btk in activation of IκB degradation in response to LPS. Its role is in transactivation by p65, occurring most likely via phosphorylation of p65 on serine 536. Our results in relation to IκB are consistent with a previous study on PBMCs isolated from control and XLA donors, who have an inactive Btk, where it was observed that LPS-induced IκB degradation was intact in XLA PBMCs, but the induction of the NFκB-dependent gene TNF was impaired (7). In addition, the general protein-tyrosine kinase inhibitor genistein, which has been shown to affect Btk (22), was shown not to inhibit LPS-activated NFκB nuclear translocation as measured by DNA binding assays but blocked the induction of IL-1β, again consistent with a lack of a role for tyrosine kinases in activating IκB degradation (23, 24).

The role of serine 536 phosphorylation in coupling p65 to coactivators, corepressors, or components of the basal tran-
Btklocalizing it to the membrane. Given our evidence for Btk involvement it is likely that the TLR4 pathway culminating in p65 phosphorylation involves both PI3K and Btk.

In conclusion our results demonstrate that in response to LPS stimulation Btk functions on the pathway leading to enhanced transactivation by p65 via phosphorylation of serine 536 but not IxBα phosphorylation and degradation. These novel findings provide an additional function for Btk and identify a new process in LPS signal transduction leading to NFκB activation.

Acknowledgments—We thank Dr. Liennhard Schmitz (German Cancer Research Center) for the Gal4-p65Δ1–331 construct. We also thank Doug Golenbock and Katherine Fitzgerald for the gift of the HEK293-TLR4 cells.

REFERENCES

1. Smith, C. I., Baskin, B., Humire-Greiff, P., Zhou, J. N., Olson, P. G., Maniar, H. S., Kjellen, P., Lambris, J. D., Christenson, B., and Hammarstrom, L. (1994) J. Immunol. 152, 557–565
2. Desiderio, S. (1997) Curr. Opin. Immunol. 9, 534–540
3. Bradley, L. A., Sweetman, A. K., Lovering, R. C., Jones, A. M., Morgan, G., Levinsky, R. J., and Kinnen, C. (1994) Mol. Hum. Genet. 3, 79–83
4. Rawlings, D. J., Saffran, D. C., Tsakada, S., Largeuaptsa, D. A., Grimaldi, J. C., Cohen, L., Mohr, R. N., Hazan, J. F., Howard, M., and Copeland, N. G. (1993) Science 261, 358–361
5. Mukhopadhyay, S., Mohanty, M., Mangla, A., George, A., Bal, V., Rath, S., and Ravindran, B. (2002) J. Immunol. 168, 2914–2921
6. Jefferies, C. A., Doyle, S., Branner, C., Dunne, A., Brint, E., Wietek, C., Walch, E., Wirth, T., and O'Neill, L. A. (2003) J. Biol. Chem. 278, 26258–26264
7. Horwood, N. J., Monahon, T. McDaid, P. J., Campbell, J. M., Mano, H., Brennan, P. F., Webster, D., and Foxwell, B. M. (2003) J. Exp. Med. 197, 1603–1611
8. McGregor, T. A., and O'Neill, L. A. (2004) Mol. Immunol. 41, 577–582
9. O'Neill, L. A. (2002) Curr. Top. Microbiol. Immunol. 270, 47–61
10. Dunne, A., and O'Neill, L. A. (2003) Sci. STKE 2003, re6
11. Brown, K., Gerstberger, S., Carlson, L., Fransson, G., and Siebenlist, U. (1995) Science 267, 1445–1448
12. Chen, Z., Hapler, M., Palombe, V. J., Melendri, P., Scherer, D., Ballard, D., and Maniatis, T. (1993) Genes Dev. 9, 1586–1597
13. Li, Q., and Verma, I. M. (2002) Nat. Rev. Immunol. 2, 725–734
14. Yang, F., Tang, E., Guan, K., and Wang, C. Y. (2003) J. Immunol. 170, 5630–5635
15. Buss, H., Durrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. (2004) J. Biol. Chem. 279, 55633–55643
16. O'Neill, L. A., and Dinarello, C. A. (2000) Immunol. Today 21, 206–209
17. Fitzgerald, K. A., Palason-McDermott, E. M., Bowie, A. G., Jefferies, C. A., Mansell, A. S., Brady, G., Brint, E., Dunne, A., Gray, P., Harte, M. T., McMurray, D., Smith, D. E., Sims, J. E., Bird, T. A., and O'Neill, L. A. (2001) Nature 413, 78–83
18. Schmitz, M. L., and Baeuerle, P. A. (1991) EMBO J. 10, 3805–3817
19. Jefferies, C. A., Bowie, A., Brady, G., Cooke, E. L., Li, X., and O'Neill, L. A. (2001) Mol. Cell. Biol. 21, 4544–4552
20. Vanden Berge, W., Plaisance, S., Boone, E., De Bosscher, K., Schmitz, M. L., Fiers, W., and Haegeman, G. (1998) J. Biol. Chem. 273, 3285–3290
21. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) J. Biol. Chem. 274, 10353–10356
22. Mukhopadhyay, S., Ramars, A. S., and Dash, D. (2001) J. Cell. Biochem. 81, 659–665
23. Yoda, B. K., Hu, J. Y., and McCall, C. E. (1996) J. Biol. Chem. 271, 18306–18309
24. Delude, R. L., Fenton, M. J., Savedra, R., Jr., Perera, P. Y., Vogel, S. N., Thieringer, R., and Golenbock, D. T. (1994) J. Biol. Chem. 269, 22253–22260
25. Schmitz, M. L., Bacher, S., and Kracht, M. (2001) Trends Biochem. Sci. 26, 186–190
26. Mansell, A., Brint, E., Gould, J. A., O'Neill, L. A., and Hertzog, P. J. (2004) J. Biol. Chem. 279, 37227–37330
27. Jiang, X., Takahashi, N., Ando, K., Otsuka, T., Tetsuka, T., and Okamoto, T. (2000) Biochem. Biophys. Res. Commun. 281, 583–590
28. Yang, E. J., Yuan, J. H., and Chung, C. K. (2004) J. Biol. Chem. 279, 1827–1837
29. Sizemore, N., Leung, S., and Stark, G. R. (1999) Mol. Cell. Biol. 19, 4798–4805
30. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254