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Inhibition of dipeptidyl aminopeptidase IV (DP-IV) by Xaa-boroPro dipeptides and use of these inhibitors to examine the role of DP-IV in T-cell function

GEORGE R. FLENTKE*, EDUARDO MUNOZ†, BRIGITTE T. HUBER†, ANDREW G. PLAUT‡, CHARLES A. KETTNER§, AND WILLIAM W. BACHOVCHIN*¶

Departments of *Biochemistry and †Pathology, Tufts University School of Medicine, Boston, MA 02111; ‡Department of Medicine, Division of Gastroenterology, New England Medical Center Hospital, Boston, MA 02111; and §Central Research and Development Department, E. I. DuPont de Nemours Company, Experimental Station, Wilmington, DE 19898

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ABSTRACT **Dipeptidyl peptidase IV (DP-IV; dipeptidyl-peptide hydrolase, EC 3.4.14.5) is a serine protease with a specificity for cleaving Xaa-Pro dipeptides from polypeptides and proteins. It is found in a variety of mammalian cells and tissues, including those of lymphoid origin where it is found specifically on the surface of CD4$^+$ T cells. Although the functional significance of this enzyme has not been established, a role in T-cell activation and immune regulation has been proposed. Here we report that Ala-boroPro and Pro-boroPro, where boroPro is the α-amino boronic acid analog of proline, are potent and specific inhibitors of DP-IV, having K_i values in the nanomolar range. Blocking the N terminus of Ala-boroPro abolishes the affinity of this inhibitor for DP-IV, while removal of the N-terminal residue, to give boroPro, reduces the affinity for DP-IV by 5 orders of magnitude. The dipeptide boronic acids exhibit slow-binding kinetics, while boroPro does not. We also report here that low concentrations of Pro-boroPro inhibit antigen-induced proliferation and interleukin 2 production in murine T-cell lines but do not inhibit the response of these T cells to the mitogen concanavalin A. These results indicate that DP-IV plays a role in antigen-induced, but not mitogen-induced, activation of T lymphocytes.**

Dipeptidyl peptidase IV (DP-IV; dipeptidyl-peptide hydrolase, EC 3.4.14.5) is a postproline cleaving enzyme with a specificity for removing Xaa-Pro dipeptides from the N terminus of polypeptides and proteins. DP-IV will also remove Xaa-Ala dipeptides from N termini, but in general this reaction is 100- to 1000-fold less efficient (1).

DP-IV is found in a variety of mammalian cells and tissues. It is most abundant in the proximal tubules of the kidneys (2, 3), intestinal epithelium (4, 5), and placenta (6). It is also found in the capillary endothelium (2), blood plasma (2), and on the cell surface of certain subsets of T lymphocytes, particularly the CD4$^+$ helper cells, although a subpopulation of human CD8$^+$ T cells also exhibits DP-IV activity (7–10). Scholz *et al.* (8) have reported that cell-surface DP-IV activity among lymphoid cells is associated with the ability of a cell to produce interleukin 2 (IL-2).

A biological function for this enzyme in mammalian systems has not yet been established. In yeast, insects, and frogs, DP-IV has been shown to be involved in the proteolytic processing of a number of bioactive peptides and proteins. These include, for example, the proteolytic processing of (*i*) an extracellular protease from Yarrowia lipolytica (11), (*ii*) the potent neurotoxin melittin from bee venom (12), (*iii*) a family of antibacterial peptides from insects referred to as cecropins (13), and (*iv*) peptides of unknown function found in frog skin that exhibit homologies with mammalian hormones and neurotransmitters (14).

In mammalian systems, a number of different biological functions for DP-IV have been proposed, ranging from digestion (15) and amino acid salvage (16), because of its occurrence in the intestine and kidney, to an involvement, through proteolytic processing, in fibronectin-mediated cell movement and adhesion (17). Because of the high frequency of Gly-Pro sequences in collagen, Hopsu-Havu *et al.* (18) suggested that this enzyme may play a role in collagen metabolism or catabolism. DP-IV in human plasma has been shown to catalyze the removal of an N-terminal Tyr-Ala dipeptide from growth hormone-releasing factor (19), and this cleavage results in the inactivation of this hormone (20).

The most intriguing of the biological functions thus far suggested for DP-IV comes from Schon *et al.* (21), who have proposed that DP-IV is involved in T-cell activation and regulation of T-cell proliferation. This proposal is based on experiments in which the addition of DP-IV inhibitors and DP-IV antibodies to cell culture systems suppressed mitogen- and alloantigen-induced T-cell proliferation, IL-2 production (22), and impaired T-cell-directed B-cell differentiation and immunoglobulin production (23, 24). Circumstantial support for this proposal comes from the observation that a number of cytokines have DP-IV susceptible Ala-Pro N-terminal sequences, as this suggests a plausible mechanism for the proposed involvement of DP-IV in T-cell activation events. These cytokines include IL-1β, IL-2, granulocyte-macrophage colony-stimulating factor, erythropoietin, and macrophage inflammatory protein 1a, recently reported to be identical to stem cell inhibitor. None of these cytokines, however, has yet been demonstrated to be a substrate for DP-IV or to have altered biological properties with the N-terminal Ala-Pro dipeptide deleted.

Inhibitors for DP-IV, more potent and specific than those now available, should prove useful in establishing the biological role or roles of this enzyme. Prospects for obtaining such inhibitors seemed particularly promising on the basis of our work with another class of post-proline-cleaving serine proteases, the IgA proteases from *Neiserria gonorrhoeae* and *Hemophilus influenzae*. Peptides containing the α-amino boronic acid analog of proline (boroPro) as the C-terminal amino acid inhibit these enzymes with K_i values in the nanomolar range (25). Here we report that dipeptides containing boroPro as the C-terminal residue are indeed potent inhibitors of DP-IV, having K_i values as low as 2×10^{-9} M and that these inhibitors are effective in inhibiting antigen-induced, but not mitogen-induced, lymphocyte proliferation

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Abbreviations: DP-IV, dipeptidyl peptidase IV; IL-2, interleukin 2; boroPro, α-amino boronic acid analog of proline; Boc, butoxycarbonyl.

¶To whom reprint requests should be addressed.

and IL-2 production in cultures of murine $CD4^+$ T-helper type 1 cells and hybridomas.

METHODS

Materials. DP-IV was purified from porcine kidneys as described (3). The enzyme activity was determined using Ala-Pro-*p*-nitroanilide as substrate, as detailed in the legend of Fig. 1. BoroPro, Ala-boroPro, Boc-Ala-boroPro (Boc, butoxycarbonyl), and Pro-boroPro were synthesized essentially as reported (25).

K_i Values. The rates of DP-IV-catalyzed hydrolysis of Ala-Pro-*p*-nitroanilide were determined at three to five concentrations of each of the boronic acid inhibitors. The reaction rates observed for Ala-boroPro (Fig. 1*B*) and for Pro-boroPro (data not shown) are nonlinear, while those observed for boroPro are linear (Fig. 1*A*). After 10 min, the nonlinear reaction rates (Fig. 1*B*) become linear. The linear portion of the rate curves can be duplicated by incubating the enzyme with Ala-boroPro or Pro-boroPro for 10 min before adding substrate. The K_i values reported in Table 1 for Ala-boroPro and Pro-boroPro were obtained from the linear rates observed after incubation of enzyme with inhibitor and thus represent final K_i values. We have not yet analyzed the nonlinear portion of the rates to obtain initial K_i values. BoroPro exhibits linear inhibition kinetics and was treated as a standard rapid equilibrium competitive inhibitor in arriving at the K_i value reported in Table 1.

The K_i values for Ala-boroPro and Pro-boroPro likely underestimate the true affinity of these inhibitors for DP-IV. Both inhibitors are unstable in aqueous solutions of neutral pH values. Ala-boroPro decomposes with a half-life between 2 and 30 min depending on pH. Pro-boroPro is substantially more stable with a half-life of at least 1.5 hr. The slow-binding inhibition kinetics of these inhibitors together with their instability in aqueous solution would combine to yield the linear rates prior to the establishment of true equilibrium between free and enzyme-bound inhibitor. K_i values obtained under these conditions must underestimate the true K_i.

Cell Lines. The murine $CD4^+$ Th-1 clone D1.1 (specific for rabbit IgG in the context of $I\text{-}A^d$) and the $CD4^+$ T-cell hybridoma 2B4 (specific for cytochrome *c* in the context of $I\text{-}A^k$) were obtained from A. Abbas (Harvard Medical School) and A. Korman (Whitehead Institute), respectively. D1.1 cells were stimulated every 2 weeks with antigen and irradiated $I\text{-}A^{d+}$ splenocytes serving as antigen-presenting cells. Supernatant from rat spleen cells that had been stimulated with Con A for 48 hr was used as a source of lymphokines. The hybridoma 2B4 was maintained at a concentration of 2×10^5 cells per ml in RPMI 1640 medium containing 10% fetal calf serum (HyClone Laboratories).

Table 1. Inhibition constants of boronic acid inhibitors of DP-IV

Inhibitor	K_i, nM
N-Boc-Ala-boroPro	>1,000,000*
BoroPro	110,000
Ala-boroPro	2
Pro-boroPro	3

*No inhibition detected.

Assays of Cell Proliferation and IL-2 Production. D1.1 cells were purified on a Ficoll/Hypaque gradient (Pharmacia) at least 2 weeks after the last stimulation with antigen. The cells were then cultured in 96-well plates, 5×10^4 cells per well, in RPMI 1640 medium supplemented with 2 mM L-glutamine, 1 mM Hepes, 50 mM 2-mercaptoethanol, antibiotics, and various amounts of Pro-boroPro. The cells were incubated for 1 hr prior to the addition of 5×10^5 irradiated $I\text{-}A^{d+}$ spleen cells plus either 200 μg of antigen per ml (rabbit IgG) or 5 μg of Con A per ml. Twenty microliters of fetal calf serum (10%) was added to each well. The cultures were incubated for 60 hr at 37°C, and [^{3}H]thymidine (0.5 μCi per well; 1 Ci = 37 GBq) was added for the final 12 hr of culture. Radioactivity incorporated into DNA was measured by liquid scintillation counting.

The 2B4 hybridoma was stimulated as described above for the D1.1 cell line, except that irradiated $I\text{-}A^{k+}$ spleen cells were used as the antigen-presenting cells and cytochrome *c* was used as antigen. The cells were incubated for 24 hr prior to assaying for IL-2 production. IL-2 production was determined in a bioassay using the HT-2 indicator cell line, which proliferates in response to IL-2. Aliquots of supernatants from the D1.1 and 2B4 cell cultures were added to cultures of HT-2 cells (10^4 cells per well) and incubated for 24 hr with [^{3}H]thymidine (0.5 μCi per well) added for the final 6 hr.

RESULTS AND DISCUSSION

Inhibition of DP-IV. Ala-boroPro, the peptide boronic acid corresponding to the classic substrate for DP-IV, is a potent

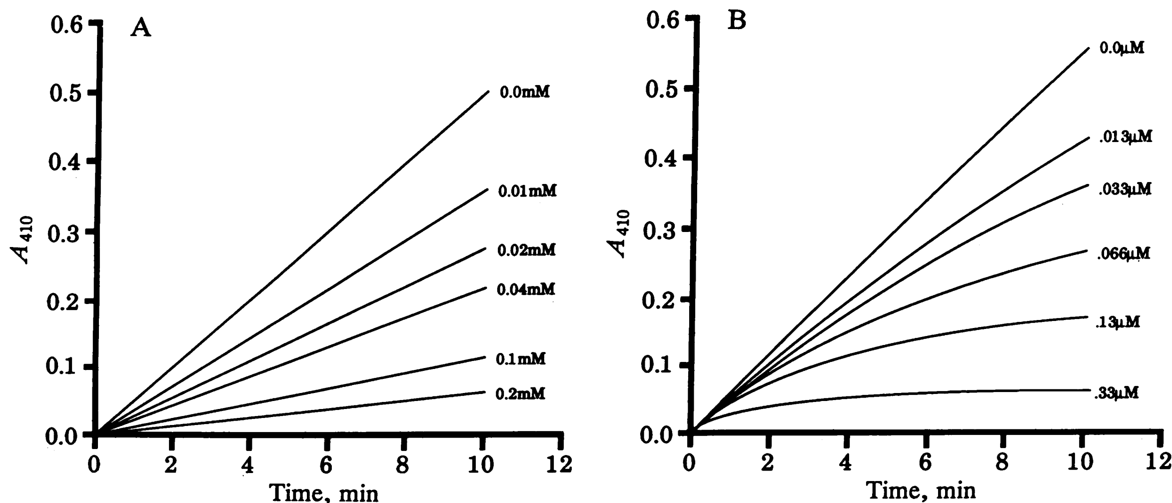

FIG. 1. Progress curves for DP-IV inhibitors. Rapid equilibrium vs. slow-binding inhibitors. The reaction solution consisted of 50 μmol of sodium Hepes (pH 7.8), 10 μmol of Ala-Pro-4-nitroanilide, 6 milliunits of DP-IV, and 2% (vol/vol) dimethylformamide in a total vol of 1.0 ml. The reaction was initiated by addition of the enzyme. Rates were measured at 25°C. The concentrations of inhibitors are shown on the graphs. (*A*) BoroPro. (*B*) Ala-boroPro.

inhibitor of DP-IV, having a K_i value of 2×10^{-9} M (Table 1). Blocking the N terminus of this inhibitor (e.g., *N*-Boc-Ala-boroPro; Table 1) abolishes the affinity, demonstrating that a free, positively charged amino group is essential for enzyme recognition and binding. The K_i of 3×10^{-9} M for Pro-boroPro demonstrates that DP-IV tolerates an imino group in place of the amino functional group on the N terminus as well as the substitution of a proline side chain in place of the alanine methyl group. This shows that the S2 specificity subsite is not highly restrictive, a result that correlates with the known substrate specificity for Xaa-Pro dipeptides (1, 26). Although DP-IV will accept nearly any amino acid at the N terminus, interactions between this amino acid and the enzyme are critical for binding. This is illustrated by the 10^5–10^6 decrease in affinity on going from Ala-boroPro or Pro-boroPro to boroPro itself (Table 1).

Fig. 1 shows that Ala-boroPro and Pro-boroPro, but not boroPro, exhibit slow-binding kinetics. This correlates with previous studies on the inhibition of other serine proteases by peptide boronic acid inhibitors, which have shown that boronic acid analogs of substrates tend to exhibit slow-binding kinetics. Boronic acid analogs of nonsubstrates or poor substrates may still be inhibitors, but they usually exhibit normal inhibition kinetics (27).

Although the inhibition kinetics reported here were carried out on DP-IV isolated from pig kidneys, we have confirmed that Pro-boroPro and Ala-boroPro inhibit DP-IV from human placenta equally well.

Effect of Pro-boroPro on T-cell Activation in Murine Lymphocyte Cell Culture Systems. Figs. 2 and 3 summarize our findings on the effects of the dipeptide boronic acid inhibitors on T-cell activation. Fig. 2 shows that Pro-boroPro is a potent inhibitor of antigen-induced (rabbit IgG) proliferation of murine $CD4^+$ T helper cells (D1.1), exhibiting a dose–response curve with 50% inhibition occurring at submicromolar concentrations of the inhibitor. In contrast, this compound is not effective in inhibiting Con A-induced proliferation even at much higher concentrations. Fig. 3 shows that Pro-boroPro is also more effective in inhibiting antigen-induced than mitogen-induced IL-2 production. A similar specific inhibition of antigen-induced IL-2 production was also observed in D1.1 cell cultures (data not shown).

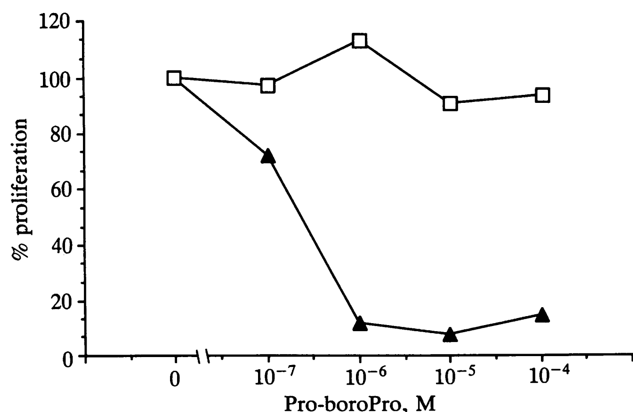

FIG. 2. Proliferative response of a murine $CD4^+$ T-helper type 1 cell line D1.1. Cells (5×10^4 per well) were cultured in the presence of 5×10^5 irradiated (2000 R; 1 R = 0.258 mC/kg) syngeneic antigen-presenting cells plus either specific antigen (▲) (rabbit IgG; 200 μg/ml) or mitogen (□) (Con A; 2 g/ml) for 3 days. The cells were pulsed during the last 6 hr of culture with [^{3}H]thymidine. The inhibitor Pro-boroPro was added at the indicated concentrations 1 hr before addition of antigen or mitogen. Cells cultured in medium only gave 425 ± 4 cpm. The results shown are of a single representative experiment, but these results have been reproduced 10 times with an SD always ≤20% in triplicate assays.

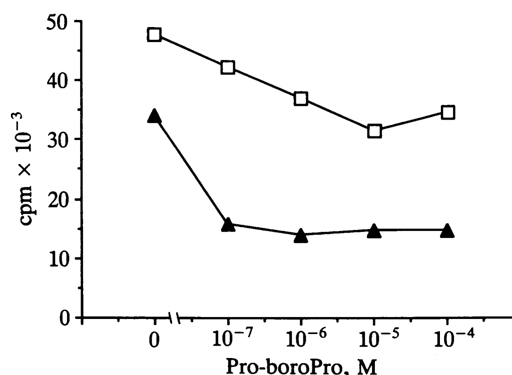

FIG. 3. IL-2 secretion by a murine $CD4^+$ T-cell hybridoma 2B4 in response to specific antigen or mitogen. Cells (5×10^4 per well) were cultured in the presence of 5×10^5 irradiated (2000 R) syngeneic antigen-presenting cells plus either specific antigen (▲) (cytochrome *c*; 5 μM) or mitogen (□) (Con A; 2 μg/ml) for 16 hr. The supernatant was then removed and tested for IL-2 content in a bioassay on HT-2 indicator cells (10^4 cells per well). The inhibitor Pro-boroPro was added at the indicated concentrations 1 hr before addition of antigen or mitogen. HT-2 cell background was 3087 ± 987 cpm.

These results were somewhat unexpected in light of the previous reports by Schon and coworkers (21–23) that DP-IV inhibitors suppress both antigen- and mitogen-induced T-cell proliferation and IL-2 production. The reasons for this discrepancy are not clear at present. However, Pro-boroPro is 4–5 orders of magnitude more potent, and probably correspondingly more specific, an inhibitor of DP-IV than those used previously. We therefore believe the results reported here to be a more reliable indicator of the role of DP-IV in T-cell function. Nevertheless, the present results lend support to the overall hypothesis proposed by Schon and coworkers (21–23) that DP-IV is involved in T-cell activation, even though they conflict with respect to the details of the role that DP-IV plays.

The concentration of Pro-boroPro needed to effect 50% inhibition of proliferation and of IL-2 production, $\approx 5 \times 10^{-7}$ M, is ≈100-fold greater than the K_i for inhibition of DP-IV by this inhibitor. This should, however, not be considered a discrepancy. The experiments illustrated in Fig. 2 required 3-day incubations. As described earlier, Pro-boroPro has a half-life of ≈1.5 hr and in these experiments only a single dose of inhibitor was given at the beginning of the incubation. Thus, the difference between K_i for enzyme inhibition and inhibition of proliferation may reflect the instability of the inhibitor. Alternatively, the total amount of DP-IV present may significantly exceed the K_i value of 10^{-9} M. Still another possible explanation is that nearly complete inhibition of DP-IV activity is necessary before suppression of T-cell activation is observed, and such complete inhibition of DP-IV would require 10^{-7} M inhibitor even if the total DP-IV concentration is small enough to ignore. It is not possible that early inhibition of DP-IV is sufficient to suppress antigen-induced proliferation, whereas longer term inhibition is necessary to suppress mitogen-induced proliferation because maintaining the inhibitor concentration at high levels by daily additions of inhibitor does not affect mitogen-induced proliferation (results not shown). Nor is it likely that the combination of antigen-stimulation plus treatment with inhibitor induces toxicity, whereas treatment with Con A and inhibitor does not because Pro-boroPro suppression of antigen-stimulated T cells can be reversed by treatment with IL-2 (results not shown). Thus, the combination of antigen and inhibitor is not toxic in these cells and there is no reason to believe that it would be to T-cell clones. Although it is well established that T-cell mitogens act through the T-cell receptor to induce proliferative signals, it is also likely that they

bind to and activate other accessory molecules, such as VLA3 and VLA5, which allows them to override the inhibitory effects of DP-IV inhibition. In contrast, antigen-induced proliferation might lack such alternative pathways.

Because of their high affinity and specificity, the dipeptide prolyl boronic acid inhibitors should prove helpful in unraveling the role played by DP-IV in T-cell activation and in defining underlying molecular mechanisms. Should DP-IV be confirmed to play the role in antigen-induced T-cell activation as the results reported here indicate, these inhibitors may also prove useful therapeutically in modifying or controlling the immune response.

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1. Heins, J., Welker, P., Schonlein, C., Born, I., Hartrodt, B., Neubert, K., Tsuru, D. & Barth, A. (1988) *Biochim. Biophys. Acta* **954**, 161–169.
2. Gossrau, R. (1985) *Histochem. J.* **17**, 737–771.
3. Wolf, B., Fischer, G. & Barth, A. (1978) *Acta Biol. Med. Ger.* **37**, 409–420.
4. Svensson, B., Danielsen, M., Staun, M., Jeppesen, L., Noren, O. & Sjostrom, H. (1978) *Eur. J. Biochem.* **90**, 489–498.
5. Corporale, C., Fontanella, A., Petrilli, P., Pucci, P., Molinaro, M. F., Picone, D. & Auricchio, S. (1985) *FEBS Lett.* **184**, 273–277.
6. Puschel, G., Mentlein, R. & Heymann, E. (1982) *Eur. J. Biochem.* **126**, 359–365.
7. Ansorge, S. & Ekkehard, S. (1987) *Acta Histochem.* **82**, 41–46.
8. Scholz, W., Mentlein, R., Heymann, E., Feller, A. C., Ulmer, A. J. & Flad, H. D. (1985) *Cell. Immunol.* **93**, 199–211.
9. Mentlein, R., Heymann, E., Scholz, W., Feller, A. C. & Flad, H. D. (1984) *Cell. Immunol.* **89**, 11–19.
10. Feller, A. C., Heijnen, C. J., Ballieux, R. E. & Parwaresch, M. R. (1982) *Br. J. Haematol.* **51**, 227–234.
11. Matoba, S. & Ogrydziak, D. M. (1989) *J. Biol. Chem.* **264**, 6037–6043.
12. Kreil, G., Haiml, L. & Suchanek, G. (1980) *Eur. J. Biochem.* **111**, 49–58.
13. Boman, H. G., Boman, I. A., Andreu, D., Li, Z., Merrifield, R. B., Schienstedt, G. & Zimmerman, R. (1989) *J. Biol. Chem.* **264**, 5852–5860.
14. Mollay, G., Vilas, U., Hutticher, A. & Kreil, G. (1986) *Eur. J. Biochem.* **160**, 31–35.
15. Morita, A., Chung, Y.-C., Freeman, H. J., Erikson, R. H., Sleisenger, M. H. & Kim, Y. S. (1983) *J. Clin. Invest.* **72**, 610–616.
16. Miyamoto, Y., Ganapathy, V., Barlas, A., Neuber, K., Barth, A. & Leibach, F. H. (1987) *Am. J. Physiol.* **252**, F670–F677.
17. Hanski, C., Huhle, T. & Reutter, W. (1985) *Biol. Chem. Hoppe-Seyler* **366**, 1169–1176.
18. Hopsu-Havu, V. K., Rintola, P. & Glenner, G. G. (1968) *Acta Chem. Scand.* **22**, 299–308.
19. Frohman, L. A., Downs, T. R., Heimer, E. P. & Felix, A. M. (1989) *J. Clin. Invest.* **83**, 1533–1540.
20. Frohman, L. A., Downs, T. R., Williams, T. C., Heimer, E. P., Pan, Y.-C. & Felix, A. M. (1986) *J. Clin. Invest.* **78**, 906–913.
21. Schon, E., Mansfeld, H. W., Demuth, H. U., Barth, A. & Ansorge, S. (1985) *Biomed. Biochim. Acta* **44**, K9–K15.
22. Schon, E., Demuth, H. U., Eichmann, E., Horst, H.-J., Korner, E.-J., Kopp, J., Mattern, T., Neubert, K., Noll, F., Ulmer, A. J., Barth, A. & Ansorge, S. (1989) *Scand. J. Immunol.* **29**, 127–132.
23. Schon, E., Jahn, S., Kiessig, S. T., Demuth, H.-U., Neubert, K., Barth, A., Von Baehr, R. & Ansorge, S. (1987) *Eur. J. Immunol.* **17**, 1821–1826.
24. Gruber, M., Scholz, W. & Flad, H. D. (1988) *Cell. Immunol.* **113**, 423–434.
25. Bachovchin, W. W., Plaut, A. G., Flentke, G. R., Lynch, M. & Kettner, C. A. (1990) *J. Biol. Chem.* **265**, 3738–3743.
26. Harada, M., Fukasawa, K., Hiraoka, B. Y., Mogi, M., Barth, A. & Neubert, K. (1985) *Biochem. Biophys. Acta* **830**, 341–344.
27. Kettner, C. A., Bone, R., Agard, D. A. & Bachovchin, W. W. (1988) *Biochemistry* **27**, 7682–7688.