Biochem. J. (1990) 266, 435–439 (Printed in Great Britain)

A continuous fluorescence displacement assay for the measurement of phospholipase A_2 and other lipases that release long-chain fatty acids

David C. WILTON
Department of Biochemistry, School of Biochemical and Physiological Sciences, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

1. A new continuous fluorescence assay for phospholipase A_2 is described which involves the displacement of the highly fluorescent fatty-acid probe 11-(dansylamino)undecanoic acid from rat liver fatty-acid-binding protein by long-chain fatty acids released as a result of phospholipase A_2-catalysed hydrolysis of phospholipids. The initial rate of decrease in fluorescence is linearly related to enzyme activity. 2. The assay will detect enzyme activity down to about 10 pmol/min per ml and gives a linear response up to about 10 nmol/min per ml. 3. The assay will work with all phospholipids that have been tested including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol and phosphatidylglycerol. Substrates carrying a net negative charge showed the highest rates of hydrolysis. 4. The assay will work, in principle, with any enzyme catalysing the release of long-chain fatty acids from a fatty-acylated substrate. This has been confirmed with pancreatic lipase and cholesterol esterase.

INTRODUCTION

The enzyme phospholipase A_2 (EC 3.1.1.4) is presumed to play a primary role in the biosynthesis of the prostaglandins, thromboxanes and leukotrienes, because it is generally accepted that the regulatory step in the formation of these vasoactive inflammatory mediators is the phospholipase A_2-catalysed release of arachidonic acid from membrane phospholipid (Flower, 1986). There is much interest in the regulation of the activity of this enzyme, particularly since it is possible that the body appears to modulate the activity of the phospholipase A_2 system via a group of glucocorticoid controlled proteins called the 'lipocortins' (Flower, 1986; Crompton *et al.*, 1988; Klee, 1988). Recently it has been demonstrated that small peptide sequences derived from the lipocortin family are also potent inhibitors of phospholipase A_2 *in vitro* and show, in parallel, anti-inflammatory potential *in vivo* (Miele *et al.*, 1988).

The precise mechanism of action of the lipocortins remains controversial, and centres around whether the proteins inhibit the system by interacting directly with the enzyme or, alternatively, by binding and sequestering the phospholipid substrate (Davidson *et al.*, 1987; Haigler *et al.*, 1987).

A limiting feature in all of these studies is the methods that are available for the assay of phospholipase A_2. At present, when high sensitivity is required, assays rely on radiochemical methods in which the release of fatty acids from labelled phospholipid is monitored. They suffer from being relatively time consuming and often lack flexibility in terms of the availability of radiochemical substrate to be hydrolysed. Alternative assays rely on the use of a much less sensitive pH-stat method to neutralize and quantify the released fatty acid.

In this paper, I describe a novel fluorescence displacement method to monitor phospholipase A_2 activity which makes use of the very high level of fluorescence enhancement (Wilkinson & Wilton, 1986) when the fluorescent probe 11-(dansylamino)undecanoic acid (DAUDA) binds to rat liver fatty-acid-binding protein (FABP). Displacement of this probe by long-chain fatty acids released as a result of phospholipase A_2 activity results in a decrease in the fluorescence signal, and the initial rate of fall of fluorescence is linearly related to enzyme activity for over a 100-fold range of enzyme concentration. The assay is sensitive down to less than 1 pkat of enzyme activity using phospholipid concentrations in the range 10–100 μM.

EXPERIMENTAL

Materials

Porcine pancreas phospholipase A_2 was obtained from Sigma and used without purification. DAUDA was obtained from Molecular Probes, Junction City, OR, U.S.A. or synthesized by treating dansyl chloride with 11-aminoundecanoic acid. Rat liver FABP was prepared by methods already described (Wilton, 1989). Dioleoylphosphatidylcholine and dioleoyl-phosphatidylglycerol were obtained from Sigma; egg phosphatidylcholine, egg phosphatidylethanolamine, phosphatidylserine from bovine spinal cord and phosphatidylinositol from wheat germ were obtained from Lipid Products, South Nutfield, Surrey, U.K. Samples provided as chloroform/methanol solutions were evaporated to dryness under N_2 and redissolved in methanol.

Assay procedure

All assays were performed in a 0.1 M-Tris/HCl buffer,

Abbreviations used: FABP, fatty-acid-binding protein; DAUDA, 11-5-(dansylamino)undecanoic acid.

pH 8, containing 0.1 M-NaCl and 2.5 mM-$CaCl_2$. A stock assay cocktail was prepared by adding to the assay buffer (20 ml) in a plastic Sterilin tube, 0.1 ml of 2 mg/ml or 10 mg/ml solutions of phospholipid in methanol followed by 0.2 ml of 0.1 mM-DAUDA in methanol. An aliquot of the assay cocktail (2 ml) was added to a 4 ml plastic fluorimeter cell and measured in a Perkin–Elmer LS3B fluorescence spectrometer. The solution was excited at 350 nm and fluorescence emission was measured at 500 nm. The fluorescence due to the DAUDA partitioning into phospholipid micelles was adjusted to zero. FABP (0.025 mg, 1.7 nmol) was added and the resulting increased fluorescence, which was normally about 10 times that obtained from the original assay mixture before being adjusted to zero, was scaled down to read about 90% on the chart recorder. In the absence of further addition, no change in this fluorescence was seen for at least 30 min using phosphatidylcholine as substrate. Samples for phospholipase A_2 were added and the fall in fluorescence was recorded using an appropriate chart speed. The fall in fluorescence was normally recorded over a period of between 1 and 10 min as appropriate, and the initial rate of fall of fluorescence was measured. Assays were performed at 25 °C.

RESULTS AND DISCUSSION

The fluorescent fatty acid probe, DAUDA, is almost non-fluorescent in buffer but, due to its hydrophobicity, it will partition into a non-polar phase and show increased fluorescence. This is the case with phospholipid micelles, in which the increase in fluorescence will be a function of micelle concentration. The probe however has a high affinity for rat liver FABP, with a K_d of less than 0.1 μM (Wilkinson & Wilton, 1987) and, as a result, in a system containing both phospholipid micelles and FABP, the DAUDA will bind preferentially to the FABP. Under the action of phospholipase A_2, long-chain fatty acids will be released as a result of phospholipid hydrolysis. These long-chain fatty acids will bind to FABP with equivalent or higher affinity than does DAUDA, and the DAUDA will be displaced with a resulting loss of fluorescence (Wilkinson & Wilton, 1987). The rate of loss of fluorescence will parallel the rate of phospholipid hydrolysis and fatty acid release. As a result of this continuous displacement and falling fluorescence, a direct measure of phospholipase A_2 activity may be obtained.

The assay system was initially evaluated using phosphatidylcholine and porcine pancreas phospholipase A_2. The general conditions used are described in the Experimental section and the molar proportions of FABP (0.85 μM), phospholipid (10–100 μM) and DAUDA (1 μM) have been optimized for maximum sensitivity.

The fluorescence traces obtained for various concentrations of phospholipase A_2 are shown in Fig. 1 using dioleoyl-phosphatidylcholine as substrate. Initial rates were reproducible and no lag was observed before the fall in fluorescence. In the absence of phospholipase A_2 or in the presence of excess EDTA to remove Ca^{2+}, the initial fluorescence reading was very stable with no detectable fall after 30 min (Fig. 1). Some variability in the overall shape of the time course was observed between apparently identical assays, probably due to the heterogeneous nature of the system; however, the initial rates were not normally affected.

A standard curve was constructed relating initial rate to enzyme concentration, and this was remarkably linear over a wide range of enzyme concentrations (Fig. 2). The upper limit of measurement with this system was dictated by the speed of the reaction and the ability of the operator to measure initial rates. Stopped-flow fluorescence may allow faster rates to be recorded.

Calibration of the assay may be readily achieved by adding known amounts of the appropriate long-chain

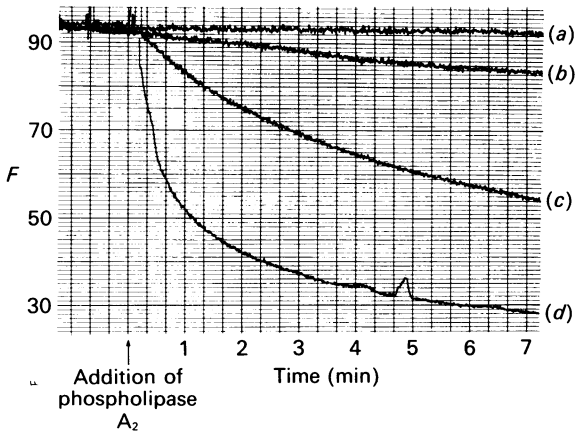

Fig. 1. Fluorescence displacement traces resulting from hydrolysis of egg phosphatidylcholine by porcine pancreas phospholipase A_2

All assays (2 ml) contained 0.05 mg of dioleoyl-phosphatidylcholine/ml, 1 μM-DAUDA and 0.0125 mg of FABP/ml. Trace (*a*) was with no addition of phospholipase A_2 or with 500 ng of enzyme in an assay without Ca^{2+} and containing 1 mM-EDTA; trace (*b*) was with 10 ng of enzyme; trace (*c*) was with 100 ng of enzyme and trace (*d*) was with 500 ng of enzyme.

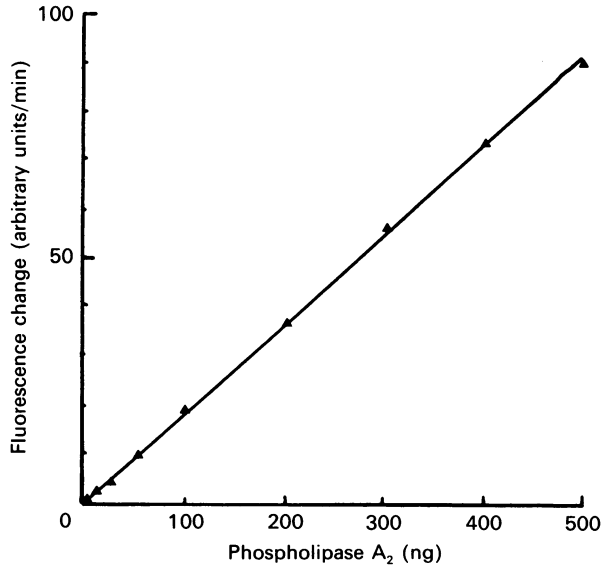

Fig. 2. Effect of phospholipase A_2 concentration on the initial rate of phosphatidylserine hydrolysis measured by fluorescence displacement

All assays (2 ml) contained 0.05 mg of phosphatidylserine/ml, 1 μM-DAUDA and 0.0125 mg of FABP/ml. Porcine pancreas phospholipase A_2 was added (5–500 ng) and the initial rate of fall in fluorescence was determined.

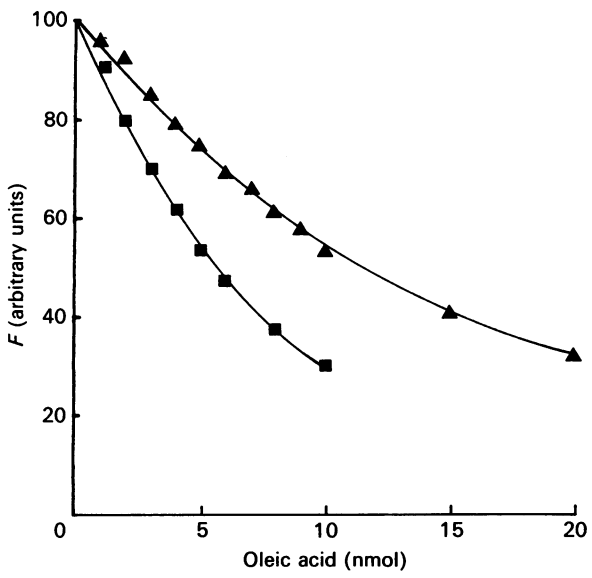

Fig. 3. Calibration of the displacement of the fluorescent probe DAUDA from FABP in the assay using oleic acid

All assays (2 ml) contained 0.05 mg of phospholipid/ml, 1 μM-DAUDA and 0.0125 mg of FABP/ml. Oleic acid (1 mM) in methanol was added in aliquots of 1.0 μl and the fall in fluorescence was determined. ▲, Egg phosphatidylcholine; ■, phosphatidylserine.

fatty acid in the absence of enzyme and measuring the quantum fall in fluorescence that results. The result of such a calibration using egg phosphatidylcholine and phosphatidylserine at 0.05 mg/ml is shown in Fig. 3. There is an essentially linear fall in fluorescence with added oleic acid for up to 50% displacement. The sensitivity of the assay, measured in terms of percentage displacement per nmol of added fatty acid, will vary according to both the concentration and nature of the phospholipid, as this will determine the partitioning of fatty acid between the phospholipid micelle and FABP. Those phospholipids possessing a net negative charge show less fluorescence enhancement due to the negatively charged DAUDA than do neutral phospholipids such as phosphatidylcholine.

In addition, differences will be observed between different long-chain fatty acids, again depending on their relative affinity for phospholipid micelles and FABP. For naturally occurring long-chain fatty acids, their abilities to displace DAUDA from FABP are relatively similar (Wilkinson & Wilton, 1987).

Previous studies with pancreatic phospholipase A_2 using phosphatidylcholine have demonstrated a pronounced lag period before the maximal rate of hydrolysis is observed (Pluckthun & Dennis, 1985; Menashe *et al.*, 1986; Lichtenberg *et al.*, 1986). However, there was not a simple explanation for this phenomenon. There is no observable lag using phosphatidylcholine or any phospholipid described in this paper as a substrate in this assay system. The kinetic properties of phospholipase A_2 have recently been reviewed (Jain & Berg, 1989).

Phosphatidylcholine micelles in the absence of a detergent are reported not to be a substrate for pancreatic phospholipase A_2 (Volwerk *et al.*, 1986), whereas obviously significant rates are observed using this fluorescent displacement assay. Calibration of the fluorescence change indicates an enzyme activity of about 50 units/mg using this assay at 25 °C and a phosphatidylcholine concentration of 13 μM. This activity is compared with a quoted value from the supplier of 600 units/mg when measured at 37 °C under standard conditions. However, standard conditions which are used for most detailed kinetic studies have involved phospholipid concentrations in the millimolar range and enzyme concentrations of the order of 0.01 mg/ml, and activity is normally measured using a pH-stat. Therefore it is difficult to compare the results from the fluorescence assay with published data; however, the conditions used in the fluorescence assay which does not contain detergent may more closely resemble those experienced within the cell.

The relatively straightforward behaviour and the activity of phospholipase A_2 when measured in this new assay system would not have been predicted from analysis of the phospholipase A_2 literature. Therefore it was necessary to determine if FABP might have a direct effect on this enzyme system. The phospholipase A_2 and phospholipid substrate were incubated together for a specific length of time in the absence of FABP followed by subsequent addition of this reagent in order to determine the amount of fatty acid that had been released during the initial incubation in the absence of protein. The data in Table 1 shows that the amount of fatty acid released was somewhat greater in the presence of FABP, indicating that FABP caused a stimulation of phospholipase activity. However, the effect of the presence of FABP on the rate was only small and not the dramatic effect needed to explain the activity of this system in the absence of anionic detergents when, according to the literature, this system should be essentially inactive.

Alternatively, because the assay is also done in the presence of a fluorescent fatty acid analogue, and it has been shown that fatty acid products may be involved in activation of the system (Pluckthun & Dennis, 1985), the effect of DAUDA on the system was also evaluated as an

Table 1. Effect of FABP and DAUDA on phospholipase A_2 activity

Assays were carried out using dioleoyl-phosphatidylcholine as substrate (0.05 mg/ml) and phospholipase A_2 (50 ng/ml). Assays were allowed to proceed for 2 min in the presence or absence of FABP (0.0125 mg/ml) and/or DAUDA (1 μM), after which time, following the addition of the omitted constituent, the fall in fluorescence was recorded. The percentage loss of fluorescence of all incubations after 2 min was calculated. Values are the means of duplicate measurements which are shown in parentheses.

Experimental condition	Loss of fluorescence after 2 min (%)	Oleic acid released in 2 min (nmol)
Control: complete assay	38.5 (37, 40)	8.0
Assay without FABP	27.0 (26, 28)	5.6
Assay without DAUDA	43.0 (45, 41)	8.9
Assay without FABP or DAUDA	24.5 (25, 24)	5.1

Table 2. Comparison of the rates of hydrolysis of different phospholipid substrates by pancreatic phosholipase A_2

All assays (2 ml) contained 0.01 mg of phospholipid/ml, 0.1 μM-DAUDA and 0.0125 mg of FABP/ml. The initial rate of fall in fluorescence was determined and the value was corrected to nmol of oleic acid released by calibration adding up to 10 nmol of oleic acid (1 mM) in methanol to the assay in the absence of phospholipase A_2. All values are means of at least three different assays using phospholipase A_2 concentrations between 2.5 and 50 ng/ml.

Phospholipid substrate	Rate of fall in fluorescence (F/min per ng of enzyme)	Specific activity (oleic acid equivalent) (μmol/min per mg)
Egg phosphatidylcholine	21	30
Dioleoyl-phosphatidylcholine	47	55
Phosphatidylethanolamine	32	43
Phosphatidylserine	143	168
Phosphatidylinositol	38	47
Dioleoyl-phosphatidylglycerol	318	374

explanation for the effectiveness of the assay. Again, no dramatic stimulation by DAUDA on the enzyme system was observed (Table 1) and hence the ability of this assay system to measure phospholipase A_2 activity is not due to the presence of either FABP or DAUDA.

Therefore the effectiveness of the fluorescence assay probably reflects the physical state and the low concentrations of substrate and enzyme that are employed, these concentrations being as much as 1000 times less than is used in some kinetic assays involving pH stat measurements. Such low concentrations of reactants would reduce the problems associated with heterogeneous systems involving lipid substrates. Thus these low concentrations of phospholipid should minimize problems with insolubility and hence maximize the effective concentration of substrate in the absence of detergents and allow significant enzyme activity to be demonstrated.

In order to verify the versatility of the assay, it was necessary to demonstrate that the system would display enzyme activity using a variety of phospholipid substrates. In Table 2 we show the results of measuring the activity of the pancreatic phospholipase using a number of different phospholipids as substrate. Assays were all performed at a fixed phospholipid concentration. The anionic phospholipid dioleoyl-phosphatidylglycerol was the best substrate under the conditions of the assay; however, of the important mammalian phospholipids, phosphatidylserine showed the highest rate of hydrolysis. The minor mammalian anionic phospholipid phosphatidylinositol showed only a marginally elevated rate of hydrolysis under these assay conditions as compared with phosphatidylcholine, whereas phosphatidylethanolamine was hydrolysed at a similar rate to phosphatidylcholine.

Comparison of the results with phosphatidylglycerol and phosphatidylinositol indicates that the size of the head group as well as phospholipid charge may be important for pancreatic phospholipase A_2 activity. However, more detailed kinetic analysis needs to be performed before definitive conclusions can be drawn. Similarly, egg phosphatidylcholine exhibited a slower rate of apparent hydrolysis than dioleoyl-phosphatidylcholine, a result which may simply reflect the nature of the fatty acid released and its capacity to displace DAUDA from FABP.

The elevated rates of hydrolysis of dioleoyl-phosphatidylglycerol as compared with dioleoyl-phosphatidylcholine observed in this study are in agreement with the results of Volwerk *et al.* (1986) who highlighted the importance of a surface negative charge at the lipid–water interface to facilitate phospholipid hydrolysis by porcine pancreas phospholipase A_2.

An important question in cell biology is the identity of the preferred phospholipid substrate(s) for the cellular phospholipases that liberate arachidonic acid. The recent availability of cloned non-pancreatic phospholipase A_2 (Kramer *et al.*, 1989; Seilhamer *et al.*, 1989) should allow a detailed analysis of the problem using this fluorescence assay.

Although the fluorescence displacement assay has been evaluated using porcine pancreas A_2, there is in principle no reason why it should not work for any source of the enzyme. The enzyme from bee venom is readily measured using this system (results not shown).

Although serum albumin is able to bind DAUDA, it does so at a site separate from the high-affinity long-chain fatty-acid binding sites of this protein, and as a result, albumin is ineffective as a substitute for FABP in this assay (D. C. Wilton, unpublished work).

General discussion

I have described a simple fluorescent displacement assay for pancreatic phospholipase A_2. The assay has a number of advantages over those described in the literature for this physiologically important enzyme. The assay is rapid and sensitive, allowing initial rate measurements to be performed within a few minutes and down to enzyme activities of about 20 pmol/min in the 2 ml assay volume. This sensitivity is not possible with methods involving a pH-stat. The assay is remarkably linear and can be used to measure initial rates up to about 20 nmol/min using a normal fluorimeter. The assay is readily calibrated by adding known amounts of the appropriate fatty acid in the absence of enzyme.

The assay is versatile and can, in principle, use any phospholipid substrate or mixture of substrates giving it an advantage over methods requiring radioactive phospholipids. Thus, for example, the effect of various phospholipid mixtures such as phosphatidylcholine and phosphatidylethanolamine on phospholipase A_2 activity may be measured using this fluorescence displacement assay. These phospholipid mixtures are known to produce complex kinetic effects (Pluckthun & Dennis, 1985) which will require detailed analysis. However, as with enzyme assays using a pH-stat, the activity recorded will only measure total fatty acid release, and a detailed study of the rate of hydrolysis of individual phospholipids in a mixture will require the use of radioactive substrates.

The method has some marginal disadvantages. (1) The method requires liver FABP and this cannot be replaced by serum albumin. (2) If high phospholipid substrate

concentrations are required, then both the FABP and DAUDA concentrations will have to be increased proportionally, and this will reduce the sensitivity of the assay. (3) If the system requires the presence of additional detergents or solvents, these will probably reduce the effectiveness of the binding of DAUDA to FABP with a corresponding loss of sensitivity. This will certainly be the case with anionic detergents such as deoxycholate which are ligands for FABP.

The assay should be particularly useful in evaluating the mechanism of inhibition of the phospholipase system by pharmacologically active compounds that have, for example, anti-inflammatory potential. This is particularly the case with the lipocortins and derived peptides, since the mechanism of inhibition by these compounds is controversial. This inhibition probably reflects the capacity of lipocortins and related proteins to bind to and to sequester phospholipid membranes rather than a direct effect on the phospholipase (Davidson *et al.*, 1987; Haigler *et al.*, 1987; Tait *et al.*, 1989). Therefore a systematic study of this problem using stoichiometric proportions of lipocortins and biologically relevant phospholipid mixtures is required.

The assay will also allow a more detailed study of the effect of cholesterol on phospholipid hydrolysis by the phospholipases, as cholesterol does not affect the binding of DAUDA to liver FABP (Wilkinson & Wilton, 1987). The effect of cholesterol on phospholipid hydrolysis is of particular interest because, like the phospholipase C-catalysed phosphatidylinositol hydrolysis, it is presumed that the important intracellular phospholipase A_2 enzymes hydrolyse phospholipid contained within the plasma membrane. It is this membrane that is the major cholesterol-containing membrane of mammalian cells (Lange *et al.*, 1989), and since cholesterol interacts with membrane phospholipids, cholesterol may have an important role in modulating the activity of these important cellular phospholipases.

Finally, this assay method can be applied in principle to measure any enzyme system which catalyses the release of free fatty acids from a substrate containing covalently bound fatty acids. Other examples include phospholipase A_1, cholesterol esterase, triacylglycerol lipase, lipoprotein lipase and enzymes that may be found that remove long-chain fatty acids from acylated proteins. Triacylglycerol lipases are of particular interest both industrially and for clinical diagnosis, so there is a major requirement for better methods of assaying this type of enzyme (Walde & Luisi, 1989). I have confirmed that the method, with slight modification because of substrate insolubility, works equally well for pancreatic triacylglycerol lipase using olive oil as substrate (D. C. Wilton, unpublished work). The method will also measure cholesterol esterase activity but with low sensitivity due to the extreme insolubility of cholesterol esters (D. C. Wilton, unpublished work).

In addition, the direct detection of total free fatty acids in some biological samples such as membrane fractions should be possible at submolar levels without the necessity for extraction, derivatization and g.l.c.

Financial support from the SmithKline (1982) Foundation for the purchase of a fluorimeter is gratefully acknowledged.

REFERENCES

Crompton, M. R., Moss, S. E. & Crumpton, M. J. (1988) Cell **55**, 1–3
Davidson, F. F., Dennis, E. A. & Glenney, J. R. (1987) J. Biol. Chem. **262**, 1698–1705
Flower, R. J. (1986) Adv. Inflammation Res. **10**, 363–366
Haigler, H. T., Schlaepfer, D. A. & Burgess, W. H. (1987) J. Biol. Chem. **262**, 6921–6930
Jain, M. K. & Berg, O. G. (1989) Biochim. Biophys. Acta **1002**, 127–156
Klee, C. B. (1988) Biochemistry **27**, 6645–6653
Kramer, R. M., Hession, C., Johansen, B., Hayes, G., McGray, P., Chow, E. P., Tizzard, R. & Pepinsky, R. B. (1989) J. Biol. Chem. **264**, 5768–5775
Lange, Y., Swaisgood, M. H., Ramos, B. V. & Steck, T. L. (1989) J. Biol. Chem. **264**, 3786–3793
Lichtenberg, D., Romero, G., Menashe, M. & Biltonen, R. L. (1986) J. Biol. Chem. **261**, 5334–5340
Menashe, M., Romero, G., Biltonen, R. L. & Lichtenberg, D. (1986) J. Biol. Chem. **261**, 5328–5333
Miele, L., Cordella-Miele, E., Facchiano, A. & Mukheerjee, A. B. (1988) Nature (London) **335**, 726–730
Pluckthun, A. & Dennis, E. A. (1985) J. Biol. Chem. **260**, 11099–11106
Seilhamer, J. J., Pruzanski, W., Vadas, P., Plant, S., Miller, J. A., Kloss, J. & Johnson, L. K. (1989) J. Biol. Chem. **264**, 5335–5338
Tait, J. F., Gibson, D. & Fujikawa, K. (1989) J. Biol. Chem. **264**, 7944–7949
Volwerk, J. J., Post, P. C., de Haas, G. H. & Griffith, O. H. (1986) Biochemistry **25**, 1726–1733
Walde, P. & Luisi, P. L. (1989) Biochemistry **28**, 3353–3360
Wilkinson, T. C. I. & Wilton, D. C. (1986) Biochem. J. **238**, 419–424
Wilkinson, T. C. I. & Wilton, D. C. (1987) Biochem. J. **242**, 485–488
Wilton, D. C. (1989) Biochem. J. **261**, 273–276

Received 18 September 1989/27 October 1989; accepted 1 November 1989