The EMBO Journal vol.4 no.11 pp.2815-2818, 1985

The phalloidin binding site of F-actin

Joël Vandekerckhove[1], Axel Deboben[2,3], Michael Nassal[2,4] and Theodor Wieland[2]

[1]Laboratory of Genetics, State University of Ghent, Ledeganckstraat 35, B-9000 Ghent, Belgium, and [2]Max-Planck-Institute for Medical Research, 29 Jahnstrasse, D-6900 Heidelberg, FRG

[3]Present address: Med. Poliklinik, 4 Vossstrasse, D-6900 Heidelberg, FRG
[4]Present address: Massachusetts Institute of Technology, Department of Chemistry, Cambridge, MA 02139, USA

Communicated by M.van Montagu

Tritium-containing affinity-labelling derivatives of phalloidin, an alkylating iodoacetyl compound (EAL) and a photolabile, carbene generating diazirine (PAL), have been reacted with rabbit muscle actin, the former after protection of thiol groups with N-ethylmaleimide. Labelled peptides generated by tryptic and/or thermolysin digestion were isolated by paper peptide mapping and characterized by determination of their amino acid sequences. EAL binds to methionine-119 and methionine-355; PAL binds to glutamic acid-117. These residues are located in regions with extremely conserved amino acid sequences. The cleft between the two domains of the actin monomer is suggested as the possible binding site for phalloidin.

Key words: phalloidin/F-actin/binding site

Introduction

Phalloidin (PHD, Figure 1) isolated from the death cap mushroom *Amanita phalloides* (Lynen and Wieland, 1937) is a bicyclic heptapeptide (for review, see Wieland, 1977; Wieland and Faulstich, 1978). It contains the uncommon amino acids 4-(*cis*)-hydroxy-L-proline (aHyp), D-threonine and, in position 7, γ,δ-dihydroxy-L-leucine. Upon acid hydrolysis it yields (two) alanine, D-threonine, *cis*-hydroxyproline, the γ-lactone of γ,δ-dihydroxy-L-leucine and, from the cross-link Ala-S-Trp (tryptathionine), β-(2-oxindol-yl-3-)alanine and L-cysteine. PHD is resistant against any proteolytic enzyme tried so far. The hydroxylated leucine side chain can be chemically modified without abolishing the biochemical property of the peptide: namely a strong acceleration of the G- to F-actin polymerization and a very specific and tight binding to F-actin (not to G-actin) (Dancker *et al.*, 1975). This interaction results in a dramatic increase in the stability of actin filaments not only towards depolymerizing conditions or agents like ultrasonication, chaotropic ions (I^-, SCN^-), DNase I, cytochalasins (Dancker *et al.*, 1975; Low *et al.*, 1975), but also towards heat denaturation and proteolytic degradation (De Vries and Wieland, 1978). A similar F-actin stabilizing effect is also observed when PHD is microinjected into cells where it seems to induce most of the previously unpolymerized actin to be incorporated into F-actin filaments and actin-related supramolecular structures (Wehland *et al.*, 1977). In addition, cellular functions thought to be associated with the organization of the microfilament system are irreversibly blocked as a result of the phalloidin-F-actin interaction (e.g., cytoplasmic streaming, cell locomotion, etc.) (Wehland *et al.*, 1978). Phalloidin exerts its stabilizing effect by reducing the critical concentration of F-actin from 10^{-6} M to 3.6×10^{-8} M (for rabbit muscle) which is also the dissociation constant of the PHD-F-actin complex (Faulstich *et al.*, 1977). Thus, the presence of two equivalents of the toxin, by mass action, decreases the concentration of actin monomers by a factor of 100. Recent kinetic studies have demonstrated that phalloidin acts primarily by preventing monomers from dissociating at both ends of the F-actin filaments (Estes *et al.*, 1981; Coluccio and Tilney, 1984). Other studies have also suggested the existence of a conformational change in the actin polypeptide chain upon interaction with phalloidin (Harwell *et al.*, 1980).

A further detailed understanding of the molecular basis of this phenomenon needs identification of the amino acid residues located in the binding region. For this purpose, F-actin was affinity labelled with an electrophilic derivative of PHD (iodo-alkyl-type; EAL) (Wieland *et al.*, 1980) (Figure 1) and a photolabile carbene-generating derivative (diazirin type; PAL) (Wieland *et al.*, 1983) (Figure 1), respectively, and the amino acid side chains involved in these cross-links were identified.

Results

In the first experiment EAL-labelled F-actin was digested with trypsin following by thermolysin, yielding two peptides (P_1 and P_2) accounting for most of the initially incorporated radioactivity (Figure 2). The presence of methionine in the hydrolysate of both P_1 and P_2 suggested that EAL was bound covalently by sulfonium salt formation. Surprisingly, these hydrolysates missed the expected phalloidin-derived amino acids (see above) and showed a simple composition which allowed us to locate readily the peptides in the actin sequence: P_1 corresponds to residues 352-356 (Phe-Gln-Gln-Met-Trp), P_2 to residues 119-121 (Met-Thr-Gln). The absence of the phalloidin-specific amino acids is probably due to a loss of aminophalloin by hydrolysis of the amide linkage between its amino group and the carboxymethyl spacer of EAL-

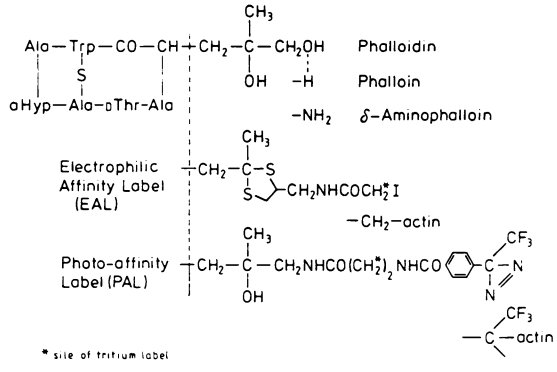

Fig. 1. The structure of phalloidin, phalloin, δ-aminophalloin and the affinity-labelled derivatives at side chain No. 7. EAL, 7{N-[^{3}H]iodoacetyl-[3-(4-aminomethyl-2-methyl-1,3-dithiolan-2-yl)-alanine]phalloidin} and PAL, N^{δ}-4-[1-azi-2,2,2-trifluoroethyl]-benzoyl-β-[2,3-^{3}H]-alanyl-aminophalloin.

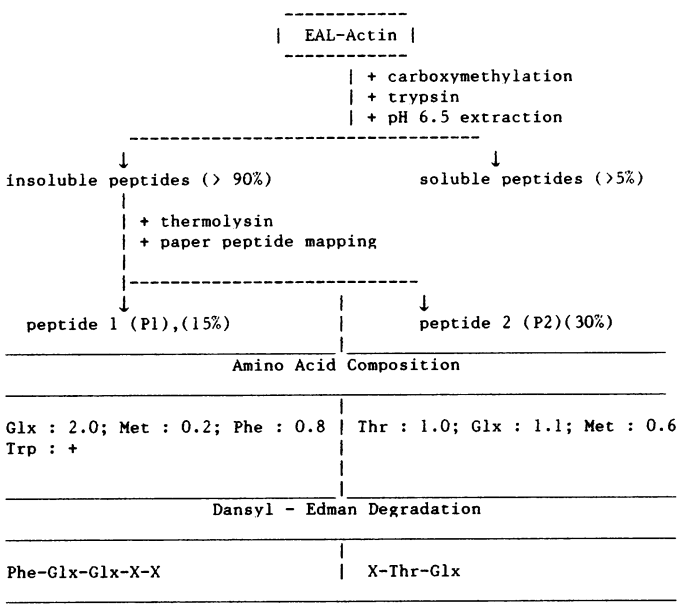

Fig. 2. Analysis of EAL-F-actin. EAL-actin was digested with trypsin. The radioactivity was only found in those peptides which were insoluble in the pH 6.5 buffer. Previous work has shown that these are peptides 119 – 147, 148 – 177 and 337 – 359 (Vandekerckhove and Weber, 1978a). The mixture was further digested with thermolysin and the radioactivity was now recovered in two soluble peptides, P_1 and P_2, which could be separated both from each other and from the unlabelled peptides by paper fingerprinting as described in Materials and methods. Aliquots of the purified radioactively labelled peptides were taken for total acid hydrolysis, determination of the specific radioactivity and determination of the partial sequence using the dansyl-Edman technique. Yields (in parentheses) were calculated after each purification step as percentage amounts of the initial radioactivity. X denotes unidentified residues.

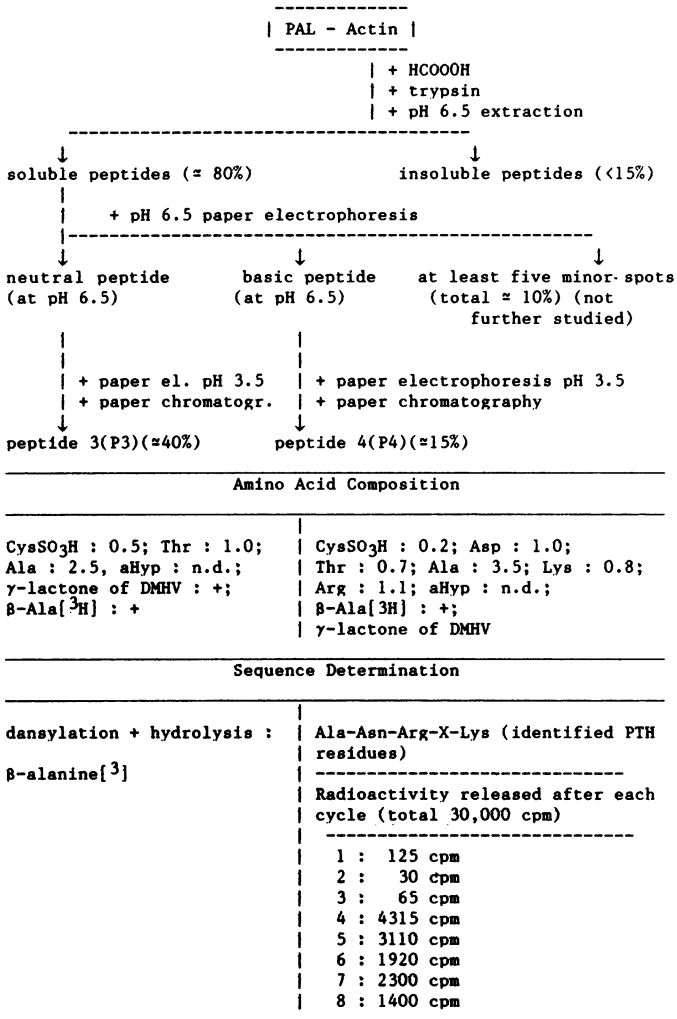

Fig. 3. Analysis of PAL-F-actin. When PAL-actin was treated with trypsin, radioactivity was detected mainly in the pH 6.5 soluble fragments (80% of the initial radioactivity), rather than the insoluble tryptic peptide mixture. Further purification yielded two major radioactive peptides, P_3 and P_4 and at least five minor fragments of which the total radioactivity did not exceed 10% of the originally incorporated radioactivity. Only peptides P_3 and P_4 were further analyzed. Their amino acid compositions were calculated from *o*-phtaldialdehyde detected residues: as a result, hydroxyproline could not be measured (n.d.). Cys SO_3H indicates cysteic acid; Hyp: hydroxyproline; β-Ala[^{3}H]: the presence of tritiated β-alanine was confirmed by separating an aliquot of the hydrolysate on cellulose thin-layer and comparison with a β-alanine reference. DMHV indicates the α, δ-diamino-γ-methylhydroxyvaleric acid; the corresponding lactone elutes from the analyzer very close to phenylalanine, it was not quantitated here. NH_2-terminal identification of P_3 was by dansylation (Bruton and Hartley, 1970) followed by hydrolysis. The amino acid sequence of peptide P_4 was determined by gas-phase sequencing. One-third of the amount of released PTH-amino acid was removed for determination of the radioactivity.

actin during digestion with trypsin and/or thermolysin, with the radiolabel attached to the methionine residue probably as the carboxymethylsulfonium ion. This also explains (i) the partial recovery of methionine upon complete acid hydrolysis and (ii) the neutral charge at pH 6.5 of both radioactive peptides in which the sulfonium cation is neutralized by the liberated S-carboxymethyl group. Peptides P_1 and P_2 were recovered in 2 and 5 nmol final yields, respectively, and their partial sequences were determined by the dansyl-Edman degradation procedure (Bruton and Hartley, 1970). This confirmed the previous allocation of the peptides. Thus, EAL binds to methionine 119 and to methionine 355 with a preference for the former of ~2-fold.

In the second experiment, F-actin was photo-affinity labelled with PAL. Unlike the former derivative, PAL is not expected to react specifically with nucleophilic amino acid residues, but may bind to all properly oriented side chains. The labelled peptides generated during tryptic digestion were purified as described in Figure 3. Only the two major peptides P_3 and P_4 were further analyzed. They account for 55% of the initial incorporated radioactivity, while 10% of the starting activity was found distributed over several minor peptides. The amino acid compositions, yields and amino acid sequences of P_3 and P_4 are given in Figure 3. P_3 was found to be N-[^{3}H]β-alanyl-δ-aminophalloin, which most likely arose from tryptic cleavage at the NH_2-terminal side of the β-alanine spacer, linking the photoactivatable group with aminophalloin (see Figure 1). Proof for this acylase activity follows from (i) the amino acid composition of P_3 showing the presence of [^{3}H]β-alanine in addition to only those amino acid residues specific to δ-amino phalloin and (ii) the identification of dansyl-β-alanine after dansylation and complete hydrolysis of P_3.

In addition to the phalloin-specific amino acid residues, peptide P_4 contained amino acid residues from the actin region 114 – 118 (Ala-Asn-Arg-Glu-Lys). This was confirmed by a sequence run with the gas-phase sequenator on 600 pmol of the peptide containing 10^5 c.p.m. of radioactivity. At each step we have identified the phenylthiohydantoin (PTH)-amino acids and also measured the released radioactivity (Figure 3). Eight cycles were carried out revealing a unique sequence fitting into region 114 – 118. No PTH derivatives could be identified at step 4 and

beyond the lysine residue at position 5. Step 4 coincides with an increase of the eluted radioactivity (Figure 3). It was therefore concluded that the photo-affinity label had reacted at Glu 117. The failure of trypsin to cleave at the COOH-terminal side of Arg 116 may be due to steric hindrance by the bulky phalloin connected with Glu 117. The continuous leakage of radioactive material past residue 4 is explained by incomplete extractability of the anilino-thiazolinone, or PTH, derivative of the phallotoxin-modified glutamic acid in the solvent systems used to transfer these compounds in the process of gas-phase sequencing. A similar observation has been made when phosphoamino acids were encountered in the course of a gas-phase sequenator run.

Discussion

Two different affinity labelling procedures have been used to covalently bind a phallotoxin to F-actin. The first reagent, EAL, reacts with two methionine residues at positions 119 and 355, respectively, with a preference for the former residue. This reaction is, as expected for alkylation of the methionine thioether, a rather slow reaction and it may be argued therefore that cross-linking occurred at unspecific sites due to denaturation of the substrate. This is very unlikely in view of (i) the known stabilizing effect of phallotoxins on actin denaturation and (ii) the fact that carboxymethylation, other than on the two methionine residues, has not taken place on the 14 additional residues of the same nature. An unexpected finding is the release of the amino-phalloin rest from labelled actin during tryptic and/or consecutive thermolysin digestion. This type of cleavage turned out to be of particular advantage, since now the phalloidin derivative functioned as an active site-directed radiolabel-transfer reagent yielding only slightly modified radioactive peptides which could readily be recognized on the basis of their amino acid composition.

A similar unexpected enzymatic cleavage has also occurred in the PAL-labelled actins. Unfortunately, here the radiolabel was situated at the unfavourable side of the cleavage site and was released from the photo-linked amino acid side chain. Since the cleavage was not complete, we also recovered a considerable fraction of uncleaved radiolabelled peptide (P_4) which allowed us to identify the cross-linked residue.

Our experiments using two types of phalloidin derivatives each having different chemical targets indicate that phalloidin binds to F-actin around residues 117 – 119 and 355. The sequences surrounding these residues exhibit a remarkable degree of sequence conservation. Indeed, visual comparison of at least 32 published amino acid and DNA sequences, representing actins from species as diverse as plants, yeast, amoeba, invertebrates and vertebrates (Collins and Elzinga, 1975; Elzinga and Lu, 1976; Vandekerckhove and Weber, 1978b, 1978c, 1979a, 1979b, 1980; Nellen *et al.*, 1981; Zakut *et al.*, 1982; Hamada *et al.*, 1982; Cooper and Crain, 1982; Fornwald *et al.*, 1982; Moos and Gallwitz, 1983; Nudell *et al.*, 1983; Hanauer *et al.*, 1983; Kost *et al.*, 1983), revealed the absence of amino acid exchanges and, in only a few cases, the existence of a limited number of conserved substitutions, explaining the general effect of the phallotoxins on actin. Interestingly, two human intronless β-actin-related pseudogenes (Moos and Gallwitz, 1983) and two soybean actin genes (Shah *et al.*, 1982, 1983) were found to encode actins with drastically altered phalloidin-binding sites involving deletions or exchanges next to, or at, the positions of the modified residues. Whereas the human actin pseudogenes are not expressed, it is not clear if the soybean actin genes are expressed. If they are, then these plant genes may encode actins with altered phalloidin binding properties.

Although our results suggest that residues 117, 119 and 355 will be surface-oriented in the F-actin-forming monomers, it is not yet clear whether these residues belong to the same monomer and, therefore, are not very far distant in the tertiary structure, or if they belong to neighbouring actin monomers. In this context it is worth mentioning recently published data obtained from X-ray studies on 6 Å resolution electron density maps of the DNase I-actin complex (Suck *et al.*, 1981). They reveal the existence of two domains in the actin monomer, a smaller one ($\sim 1/3$ of the molecule) comprising the amino-terminal part and a larger one possibly with the carboxyl-terminal part, with ATP (or ADP) being located in the cleft between the two domains. A conformational change is known to occur in the ATP-binding region during the transition of G- to F-actin, since photolabelling with 8-azido-ATP revealed a close proximity of the nucleotide to Lys 336 and Trp 356 in G-actin but only to Lys 336 in the F-actin monomer (Hegyi *et al.*, 1985). This means that Trp 356, together with Met 355, must have been translocated during this transition. One may suggest that by such a conformational change the Met 355 side of the interdomain cleft becomes accessible to the phallotoxin that stabilises the altered structure in the filament. Amino acids Glu 117 and Met 119 that likewise react with the phallotoxin labels, probably reside in the small domain and could be located at the opposite flank of the cleft suggesting an intercleft position as the binding site of F-actin for phalloidin.

Materials and methods

Rabbit skeletal muscle actin was purified as described (Dancker and Hoffmann, 1973; Mannherz *et al.*, 1980).

Preparation of EAL-F-actin

F-actin (1 mg/ml F-buffer: 10 mM Tris-HCl, 100 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, pH 7.4) was affinity labelled by incubation with a 5-fold molar excess of EAL (sp. act. 240 mCi/mmol) for 36 h at 37°C; [in order to avoid unspecific alkylation of the potentially reactive cysteine residues in F-actin (Lusty and Fasold, 1968), the protein was previously allowed to react with a 100-fold excess of N-ethylmaleimide for 1 h at room temperature]. The labelling reaction was terminated by addition of 2 mg β-mercaptoethanol per ml of protein solution followed by centrifugation for 1 h at 130 000 *g*. 10 mg of the radiolabelled F-actin pellet (sp. act. 2×10^{10} c.p.m./nmol) were reduced and carboxymethylated in the presence of urea with iodoacetic acid (Crestfield *et al.*, 1963) in order to block remaining free SH-groups.

Preparation of PAL-F-actin

F-actin (1.6 mg; 35 nmol) in 4.45 ml F-buffer was incubated with 43 μg (39.2 nmol) of PAL (sp. act. 25 Ci/mmol) for 1 h at ambient temperature in the dark in a 10-ml Pyrex screw-cap glass tube (filter for wavelengths >300 nm). Argon was slowly bubbled through the solution and the sample was irradiated for 10 min with a high-pressure mercury lamp (Nassal, 1983). The non-covalently bound analogue was displaced by addition of a 33-fold excess of phalloidin dissolved in 900 μl H_2O and allowed to exchange for 1 h, after which F-actin was collected by centrifugation at 120 000 *g* for 1 h. The ratio of radioactivity in the pellet versus the supernatant indicated a cross-linking efficiency of 22%. The tritiated affinity labelled F-actin was mixed with 5 mg of non-tritiated derivative, prepared as described above with non-radioactive PAL, performic acid oxidized (Hirs, 1956), dialyzed against water, then against 0.5% NH_4HCO_3, and finally digested with trypsin.

Methods used for analysis of derivatized actins

Derivatized actins were digested for 2 h with trypsin in 0.5% NH_4HCO_3 buffer (enzyme-substrate ratio: 1/60; by weight) and lyophilized. Peptides were first separated into a pH 6.5 (10% pyridine; 0.5% acetic acid), soluble and insoluble fraction. The soluble peptides were immediately separated while the insoluble peptides mixture was further fragmented with thermolysin before separation. Peptides were purified on paper by a combination of electrophoresis at pH 6.5, pH 3.5 and descending chromatography in butan-1-ol, acetic acid, water, pyridine (15:3:12:10; by volume) (Vandekerckhove and Weber, 1978a). Peptides were detected by a dilute fluorescamine stain (Vandekerckhove and Van Montagu, 1974) and tritiated peptides by fluorography at −80°C. For this purpose, the paper was dipped in a 20% solution of 2,5-diphenyloxazole in toluene (Bonner and Stedman, 1978). The scintillator was removed by extensive washing of the

paper strips with toluene, and the peptides recovered by elution with pH 6.5 buffer. Acid hydrolysis was carried out in 6 N HCl for 24 h at 110°C and the hydrolysate was analyzed on a Biotronik amino acid analyzer after reaction with *o*-phtaldialdehyde (Benson and Hare, 1975). This latter reagent does not allow detection of proline and hydroxyproline. Amino acid sequences of the labelled peptides were determined either by the manual dansyl-Edman degradation procedure (Bruton and Hartley, 1970) or with the gas-phase sequenator operated as described by Hewick *et al.* (1981). PTH-amino acids were identified by h.p.l.c. analysis as described by Hunkapiller and Hood (1983). One-third of the amount of released PTH-amino acid was removed for the determination of the radioactivity. Tryptophan was identified by Ehrlich reaction on paper (Penke *et al.*, 1974).

Acknowledgements

We acknowledge the excellent technical assistance of M.Puype and J.Van Damme. J.V. is Research Associate of the Belgian National Fund for Scientific Research (N.F.W.O.). The work was supported by the N.F.W.O., the Volkswagen Stiftung and, in part, by the Deutsche Forschungsgemeinschaft (D.F.G.).

References

Benson,J.R. and Hare,P.E. (1975) *Proc. Natl. Acad. Sci. USA*, **72**, 619-622.
Bonner,W.M. and Stedman,J.D. (1978) *Anal. Biochem.*, **60**, 35-50.
Bruton,C.J. and Hartley,B.S. (1970) *J. Mol. Biol.*, **52**, 165-173.
Collins,J.H. and Elzinga,M. (1975) *J. Biol. Chem.*, **250**, 5915-5920.
Coluccio,L.M. and Tilney,L.G. (1984) *J. Cell. Biol.*, **99**, 529-535.
Cooper,A.D. and Crain,W.R., Jr. (1982) *Nucleic Acids Res.*, **10**, 4081-4092.
Crestfield,A.M., Moore,S. and Stein,W.H. (1963) *J. Biol. Chem.*, **238**, 622-627.
Dancker,P. and Hoffmann,M. (1973) *Z. Naturforsch.*, **28**, 401-421.
Dancker,P., Low,I., Hasselbach,W. and Wieland,T. (1975) *Biochim. Biophys. Acta*, **400**, 407-414.
De Vries,J. and Wieland,T. (1978) *Biochemistry (Wash.)*, **17**, 1965-1968.
Elzinga,M. and Lu,R.C. (1976) in Perry,S.V., Margreth,A. and Adelstein,R.S. (eds.), *Contractile Systems in Non-Muscle Tissues*, Elsevier, North Holland, Amsterdam, Netherlands, pp. 29-37.
Estes,J., Selden,L. and Gershman,L. (1981) *Biochemistry (Wash.)*, **20**, 708-712.
Faulstich,H., Schäfer,A.J. and Weckauf,M. (1977) *Hoppe-Seyler's Z. Physiol. Chem.*, **358**, 181-186.
Fornwald,J.A., Kuncio,G., Peng,T. and Ordahl,C.P. (1982) *Nucleic Acids Res.*, **10**, 3861-3876.
Hamada,H., Petrino,M.G. and Kankunaga,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 5901-5905.
Hanauer,A., Levin,M., Heilig,R., Daegelen,D., Kahn,A. and Mandel,J. (1983) *Nucleic Acids Res.*, **11**, 3505-3516.
Harwell,D.O., Sweeney,M.L. and Kirkpatrick,F.H. (1980) *J. Biol. Chem.*, **255**, 1210-1220.
Hegyi,G., Szilagyi,L. and Elzinga,M. (1985) *J. Muscle Res. Cell Motil.*, in press.
Hewick,R.M., Hunkapiller,M.W., Hood,L.W. and Dreyer,W.J. (1981) *J. Biol. Chem.*, **256**, 7990-7997.
Hirs,C.H.W. (1956) *J. Biol. Chem.*, **219**, 611-621.
Hunkapiller,M.W. and Hood,L.W. (1983) *Methods Enzymol.*, **91**, 486-493.
Kost,T., Theodorakis,N. and Hughes,S.H. (1983) *Nucleic Acids Res.*, **11**, 8287-8301.
Low,I., Dancker,P. and Wieland,T. (1975) *FEBS Lett.*, **54**, 263-265.
Lusty,C. and Fasold,H. (1968) *Biochemistry (Wash.)*, **8**, 2933-2939.
Lynen,F. and Wieland,U. (1937) *Justus Liebigs Ann. Chem.*, **533**, 93-117.
Mannherz,H.G., Goody,R.S., Konrad,M. and Nowak,E. (1980) *Eur. J. Biochem.*, **104**, 367-379.
Moos,M. and Gallwitz,D. (1982) *Nucleic Acids Res.*, **10**, 7843-7849.
Moos,M. and Gallwitz,D. (1983) *EMBO J.*, **2**, 757-761.
Nassal,M. (1983) *Justus Liebigs Ann. Chem.*, **1983**, 1510-1523.
Nellen,W., Donath,C., Moos,M. and Gallwitz,D. (1981) *J. Mol. Appl. Genet.*, **1**, 239-244.
Nudell,U., Zakut,R., Shani,M., Neuman,S., Levy,Z. and Yaffe,D. (1983) *Nucleic Acids Res.*, **11**, 1759-1771.
Penke,B., Ferenczi,R. and Kovacs,K. (1974) *Anal. Biochem.*, **60**, 45-50.
Shah,D.M., Hightower,R.C. and Meagher,R.B. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1022-1026.
Shah,D.M., Hightower,R.C. and Meagher,R.B. (1983) *J. Mol. Appl. Genet.*, **2**, 111-126.
Suck,D., Kabsch,W. and Mannherz,H.-G. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4319-4323.
Vandekerckhove,J. and Weber,K. (1978a) *Eur. J. Biochem.*, **90**, 451-452.
Vandekerckhove,J. and Weber,K. (1978b) *Proc. Natl. Acad. Sci. USA*, **75**, 1106-1110.
Vandekerckhove,J. and Weber,K. (1978c) *Nature*, **267**, 720-721.
Vandekerckhove,J. and Weber,K. (1979a) *Differentiation*, **14**, 123-133.
Vandekerckhove,J. and Weber,K. (1979b) *FEBS Let.*, **102**, 219-222.
Vandekerckhove,J. and Weber,K. (1980) *Nature*, **284**, 457-477.
Wehland,J., Osborn,M. and Weber,K. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5613-5617.
Wehland,J., Stockem,W. and Weber,K. (1978) *Exp. Cell. Res.*, **115**, 451-454.
Wieland,T. (1977) *Adv. Enzyme Regul.*, **15**, 285-300.
Wieland,T. and Faulstich,H. (1978) *CRC Crit. Rev. Biochem.*, **5**, 185-260.
Wieland,T., Deboben,A. and Faulstich,H. (1980) *Justus Liebigs Ann. Chem.*, **1980**, 416-424.
Wieland,T., Hollosi,M. and Nassal,M. (1983) *Justus Liebigs Ann. Chem.*, **1983**, 1524-1533.
Zakut,R., Shani,M., Givol,D., Neumann,S., Yaffe,D. and Nudel,U. (1982) *Nature*, **298**, 857-859.

Received on 26 July 1985