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Selective Carbethoxylation of the Histidine Residues of Actin by Diethylpyrocarbonate

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Histidine residues of actin were carbethoxylated with diethylpyrocarbonate. Four and three histidine residues per actin monomer were rapidly carbethoxylated in the G and F form, respectively. After partial carbethoxylation of G-actin a non-polymerizable fraction could be separated by ultracentrifugation. The analysis of these fractions showed that one of the four fast-reacting histidines of G-actin plays an essential role in polymerization.

Removal of the carbethoxy groups from the histidine residues by hydroxylamine treatment restored the ability to polymerize.

The specificity of the reaction was tested by the use of [^{14}C]diethylpyrocarbonate.

After tryptic digestion of non-polymerizable carbethoxylated actin a single histidyl peptide was isolated by gel filtration and diagonal electrophoresis. The histidine residue essential for polymerization was identified as histidine-40.

The essential role of histidine residues in the polymerization of actin has been observed by several investigators in photooxidation experiments [1–4].

We have shown earlier [5] that the ability of G-actin to polymerize considerably decreases on carbethoxylation of histidine residues, whereas carbethoxylated F-actin is not depolymerized spontaneously, and carbethoxylated G-actin obtained by depolymerization of diethylpyrocarbonate-treated F-actin can be polymerized. The number of histidine residues involved in the polymerization process was not determined in these experiments.

In the present work non-polymerizable actin was separated from the polymerizable fraction after partial carbethoxylation of G-actin to identify the histidine residues whose blocking prevents polymerization.

The specificity of the reaction between diethylpyrocarbonate and histidyl group(s) was established by using [^{14}C]diethylpyrocarbonate. This seemed necessary because according to Melchior and Fahrney [6] diethylpyrocarbonate also reacted with other amino acid side chains even in a slightly acidic medium.

MATERIALS AND METHODS

Actin was extracted from acetone-dried muscle powder prepared by the method of Bátány *et al.* [7]. The powder was extracted with 0.2 mM ATP, 2 mM mercaptoethanol at 0 °C and the G-actin was polymerized by the addition of KCl to 50 mM final concentration. For further purification Mommaerts' method [8] was used.

Myosin was prepared according to Portzehl *et al.* [9] and was used after the removal of impurities by sedimentation at $105000 \times g$ for 1 h.

Protein content was measured by the biuret method of Gornall *et al.* [10] or spectrophotometrically from the absorbances at 280 and 260 nm.

Diethylpyrocarbonate was a commercial product of Bayer (Baycovin). [^{14}C]Diethylpyrocarbonate (1 mCi/mmol) was obtained from the Institute of Isotopes of the Hungarian Academy of Sciences. All other chemicals were reagent grade products from Reanal (Budapest).

For carbethoxylation, diethylpyrocarbonate dissolved in absolute ethanol was added to a solution containing 4.5 mg/ml G-actin and 5 mM acetate buffer pH 6.5. The mixture was incubated at 0 °C for 12 h. Over this period the excess of diethylpyrocarbonate decomposes to ethanol and CO_2. G-actin was then polymerized by the addition of KCl to 0.1 M final concentration. A portion of this mixture was centrifuged at $105000 \times g$ for 2 h in order to separate polymerized and non-polymerized actin.

The number of carbethoxy-histidine groups formed was evaluated by the method of Ovádi *et al.* [11] on the basis of the absorbance change at 240 nm ($\Delta\varepsilon = 3200\ M^{-1} \times cm^{-1}$).

Enzyme. ATPase (EC 3.6.1.3).

For the removal of the carbethoxy groups from the histidine residues, the samples were dialysed against 1000 vol. 0.01 M neutralized hydroxylamine for 12 h, or by direct addition of hydroxylamine to 0.1 M final concentration, as indictaed in the text.

Viscosity measurements were carried out in an Ostwald viscosimeter at 0 °C at 1 mg/ml actin concentration.

The ATPase activity of synthetic actomyosin was measured at 20 °C for 5 min in the following reaction mixture: 0.6 mg/ml myosin, 0.1 mg/ml actin, 2 mM ATP, 2 mM $MgCl_2$, 20 mM Tris-maleate buffer pH 7.4, final volume 2 ml.

The tryptic digestion of carbethoxylated actin was carried out in a Radiometer pH-Stat at pH 7.2 for 6 h in the presence of 5 mM EDTA and 5 mM mercaptoethanol. Trypsin (2× crystallized, Serva) was added in a 1:60 (w/w) ratio to actin, three times at intervals of 2 h as proposed by Johnson *et al.* [12]. During tryptic digestion the radioactivity of [^{14}C]-carbethoxylated actins decreased only by 5–10 % as measured on lyophilized samples.

The tryptic digests were fractionated in the cold on a Sephadex G-25 column (2×200 cm) equilibrated with 0.05 M ammonium acetate buffer pH 6.

The peptide containing the carbethoxy histidine was isolated by diagonal electrophoresis as described by Sajgó [13] and its amino-acid composition was determined after hydrolysis with 6 N HCl for 24 h at 105 °C in a Jeol JAH-6 automatic analyser by the single-column method of Dévényi [14]. The N-terminal residue was determined by the dansyl method of Gray and Hartley [15] and the dansyl amino acids were identified by the electrophoretic method of Sajgó [16].

RESULTS

Determination of Fast-Reacting Histidine Residues

The time course of the reaction between the histidine residues and diethylpyrocarbonate cannot be described by a simple kinetic formula because of the simultaneous hydrolysis of the reagent. On the other hand, Melchior and Fahrney [6] observed that the reaction in dilute solution can be characterized by the equation

$$\log \frac{G}{G_0} = \frac{k_G [\mathrm{R}]_0}{k_{\mathrm{HOH}}}$$

where G_0 = the number of reactive groups in the protein, G = the number of reactive groups not reacted until time ∞ (after completion of the reaction), k_G = the rate constant of carbethoxylation of the reactive groups, k_{HOH} = the pseudo-first-order rate constant of diethylpyrocarbonate hydrolysis and $[\mathrm{R}]_0$ = the initial concentration of the reagent.

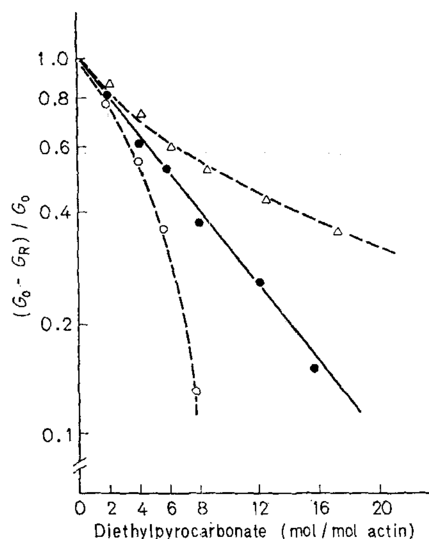

Fig. 1. *Determination of the number of histidine residues in G-actin which react rapidly with diethylpyrocarbonate.* 0.1 µmol/ml G-actin was carbethoxylated with 2–20 mol equivalents of diethylpyrocarbonate at 0 °C, pH 6.5 for 12 h. Immediately before carbethoxy-histidine measurement the samples were ten-fold diluted. The ratios $(G_0-G_R)/G_0$, where G_0 = number of reactive histidines and G_R = number of histidine residues which have reacted with diethylpyrocarbonate, have been plotted logarithmically as a function of diethylpyrocarbonate concentration applied for various assumed values of G_0. (○----○) $G_0 = 3$; (●——●) $G_0 = 4$; (△----△) $G_0 = 5$

On the basis of this formula one can determine the number of fast-reacting residues of approximately identical reactivities in a system where groups of different reactivities exist and the rate constants are sufficiently different.

In this case log (G/G_0) is plotted against $[\mathrm{R}]_0$ at various plausible values of G_0, and the true value is regarded as that which gives a straight line. For this reason carbethoxylation of G and F-actin, as well as of α-*N*-acetylhistidine was measured as a function of diethylpyrocarbonate concentration after completion of reaction.

The values of log $(G_0-G_R)/G_0$, where G_0 = number of reactive histidine residues in actin and G_R = number of histidine residues which have reacted with diethylpyrocarbonate, was calculated for various possible of G_0 and plotted against the added quantities of diethylpyrocarbonate. The results for G-actin and F-actin are shown in Fig. 1 and 2, respectively.

It can be seen that for G-actin $G_0 = 4$ while for F-actin $G_0 = 3$ yielded straight lines. With other values significant deviations from the straight line were obtained. On the basis of this formula it cannot be excluded that one residue reacts faster than the others, because the static equation of Melchior and Fahrney is unsuitable to calculate correct kinetic

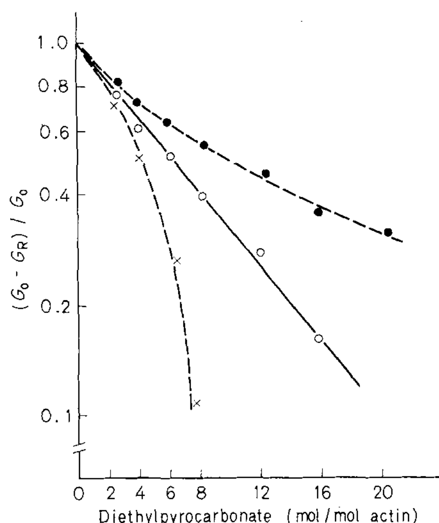

Fig. 2. *Determination of the number of histidine residues in F-actin which react rapidly with diethylpyrocarbonate.* 0.1 µmol/ml F-actin was carbethoxylated with 2–20 mol equivalents of diethylpyrocarbonate at 0 °C, pH 6.5 for 12 h in the presence of 0.1 M KCl. Before carbethoxy-histidine measurement the samples were diluted ten-fold. Results were plotted as described in Fig. 1. (×----×) $G_0 = 2$; (○——○) $G_0 = 3$; (●----●) $G_0 = 4$

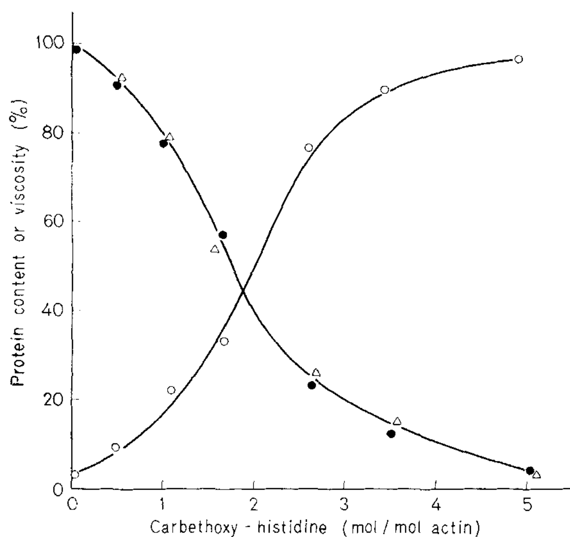

Fig. 3. *Actin content in the pellet and supernatant fractions after centrifugation of carbethoxylated G-actin polymerized in KCl.* G-actin was carbethoxylated as described in Methods and centrifuged at 105000×*g* after the addition of 0.1 M KCl. Protein content or viscosity (log η_{rel}) is expressed as a percentage of the untreated control. (○) Protein content of the supernatant; (●) protein content of the pellet; (△) viscosity of non-centrifuged samples

data. However, from our other experiments (Fig. 3) it seems that the reactivities of the readily accessible histidines are nearly equal, therefore an approximate calculation of the rate constant of the fastest reacting residues seems to be justifiable (see Discussion).

Determination of Histidine Residues Essential for Polymerization

If a protein contains several groups of the same or nearly the same reactivity and one or more of these groups are functionally essential, their blocking by chemical modification can lead to a gradual alteration of functional properties. The number of residues responsible for inactivation is then difficult to determine since the system may contain a mixture of active and inactive molecules modified to the similar extent. For this reason an attempt was made to separate the inactive molecules from the active ones after different degrees of modification. G-actin sample carbethoxylated to different degrees were polymerized with 0.1 M KCl and centrifuged for 2 h at 105000 ×*g*. The carbethoxy-histidine and protein contents were then measured both in the supernatant, *i.e.* the non-polymerizable actin, and in the pellet (Fig. 3).

The protein content in the supernatant fraction increases with increasing diethylpyrocarobnate concentration. It was found that about 25% of actin contained in the sample becomes non-polymerizable after blocking of a single histidine residue and that practically no polymerizable actin (pellet) is obtainable after the carbethoxylation of more than four histidine residues.

From the samples treated with low diethylpyrocarbonate concentrations non-polymerizable actin could be isolated, in which only one mole carbethoxy-histidine was detected.

In the case of partial carbethoxylation, when carbethoxylated but polymerizable actin could be isolated also, in the supernatant only the suitably modified molecules were selected by ultracentrifugation. These data suggest that only one of four accessible histidine residues caused the inhibition of polymerization. As polymerization is fully inhibited only after blocking of four fast-reacting residues, it can be concluded that these reacted in a random manner.

The viscosity of the non-centrifuged carbethoxylated samples in 0.1 M KCl decreased parallel with the quantity of polymerizable actin. This shows that the inactive molecules were indeed separated from the active ones.

To exclude the possibility that in addition to the histidyl group the modification of another residue was also involved in the loss of polymerizability, the experiment was repeated with ^{14}C-labelled diethylpyrocarbonate. Non-polymerizable actin was separated after partial carbethoxylation. The radioactivity measurements showed 1.34 mol carbethoxy group/mol actin. Spectrophotometrically the carbethoxy-histidine content of the sample was 1.1 mol/mol actin. According to Melchior and Fahrney [6] the sample was dialyzed against 0.01 M hydroxylamine for 12 h in order to remove the carbethoxy groups from histidines. The ^{14}C radioactivity measured after

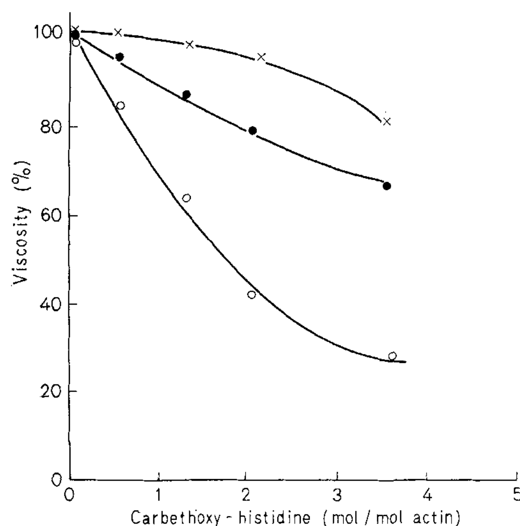

Fig.4. *Reversibility of the inhibition of polymerization.* G-actin was carbethoxylated and the carbethoxylated samples were treated with hydroxylamine as described in Methods. The viscosity of the samples polymerized in 0.1 M KCl was measured before and after hydroxylamine treatment. The viscosity (log η_{rel}) is expressed as a percentage of the untreated control. (○) Viscosity before hydroxylamine treatment; (●) viscosity 2 h after hydroxylamine treatment; (×) viscosity 18 h after hydroxylamine treatment

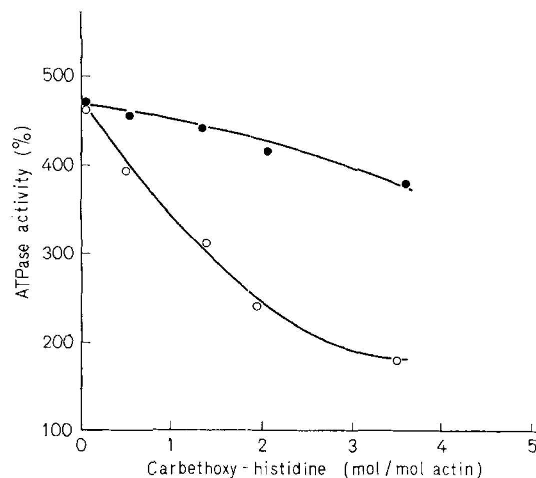

Fig.5. *Reversibility of the myosin ATPase activation.* Actin was carbethoxylated and treated with hydroxylamine and the ATPase activity was measured as described in Methods. ATPase activity is expressed as a percentage of myosin ATPase in the absence of actin. (○) ATPase activity of myosin activated by carbethoxylated actin before hydroxylamine treatment; (●) ATPase activity of myosin activated by carbethoxylated actin 18 h after hydroxylamine treatment

dialysis showed that only 0.14 mol carbethoxy group/mol actin was not removed by hydroxylamine. These carbethoxy groups are probably bound to amino groups. Since the amount of the latter is very small, whereas that of the carbethoxy histidines is about 1 mol/mol actin, it can be reasonably assumed that the loss of ability to polymerize is due to the carbethoxylation of a single histidyl residue.

Further evidence for this is provided by the recovery of ability to polymerize on treatment of carbethoxylated actin with hydroxylamine (Fig.4). The viscosity (ability to polymerize) of actin samples carbethoxylated to various degrees was measured before and 2 or 18 h after the addition of hydroxylamine to 0.1 M final ocncentration. The percentage change of viscosity, relative to the untreated control, is plotted against the number of carbethoxylated histidines before and after the removal of the carbethoxy group. The ability of slightly modified actin samples to polymerize could be fully restored but even after the carbethoxylation of 3.5 mol histidine/mol actin the inhibition could be reversed to 80%.

The reversibility also applied to the activating effect of actin on myosin ATPase (Fig.5). The activation by actin must be due to the recovery of the ability to polymerize since no change was obtained in the ATPase activating effect of partially carbethoxylated and polymerizable actin fractions. Consequently, the accessible histidyl residues which are not involved in polymerization do not play any role in the actin-myosin interaction either.

Identification of the Peptide Containing the Histidyl Residues Essential for Actin Polymerization

G-actin was modified with [^{14}C]diethylpyrocarbonate to obtain 1—1.5 mol carbethoxyhistidine per mole actin. The partially carbethoxylated G-actin was polymerized by the addition of 0.1 M KCl and the polymerized actin was separated from the non-polymerizable fraction by centrifuging at $105000 \times g$ for 2 h. The two fractions were separately digested by trypsin. The tryptic digests were lyophilised and fractionated on a Sephadex G-25 column. The radioactivities of the fractions are shown in Fig.6.

In the elution profile of the tryptic peptides derived from non-polymerizable actin only one fraction had high ^{14}C activity. However two other peaks appeared if the extent of modification surpassed 1 mol carbethoxy-histidine/mol actin.

No peak corresponding to the second (highest peak) of non-polymerizable actin appears in the radioactivity profile of the digest of the polymerizable sample which exhibited measurable activities only in the two other fractions. This fact indicates that the second peak of non-polymerizable actin contains the histidyl residue responsible for polymerization and that this histidine is not carbethoxylated in polymerizable actin.

The peptide containing the essential histidine was isolated by diagonal electrophoresis from the non-

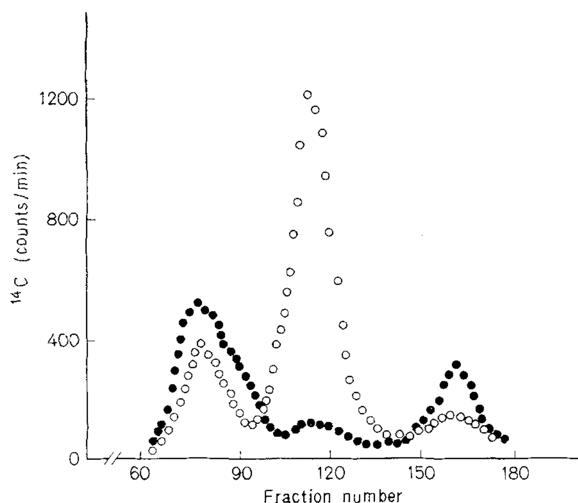

Fig. 6. *Elution profiles of tryptic digests of polymerizable and non-polymerizable fractions of G-actin carbethoxylated with [^{14}C]diethylpyrocarbonate.* Carbethoxylated G actin containing 1.5 mol carbethoxy-histidine per mole actin was polymerized and separated by ultracentrifugation. Both the supernatant and the pellet were digested by trypsin as described in Methods. Tryptic digests were fractionated subsequently on the same Sephadex G-25 column. (●) ^{14}C in the pellet fractions; (○) ^{14}C in the supernatant fractions

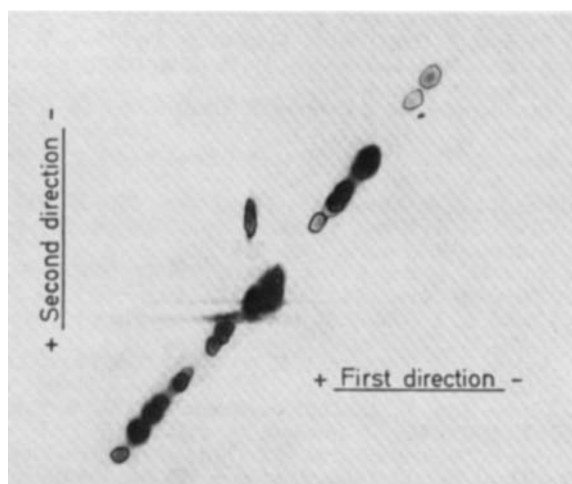

Fig. 7. *Diagonal electrophoretogram of the peptide fraction containing the essential histidyl residue.* Electrophoresis was carried out in both directions at pH 5 on Whatman 3-MM paper. The electrophoretogram was developed with ninhydrin

polymerizable actin fraction with the highest activity (Fig. 7).

The first electrophoresis was carried out on Whatman 3-MM paper at pH 5 and 35 V/cm for 2 h in a horizontal plate, tap-water-cooled apparatus.

After the first run carbethoxy groups were removed from the imidazole groups by exposing the paper to ammonia vapour for 12 h at room temperature.

The a strip of the electrophoretrogram was sewn to another sheet of Whatman paper and a second run was made perpendicular to the first electrophoresis.

Since the imidazole group recovered its positive charge on removal of the carbethoxy group, in the second run only the peptide containing the modified histidine was displaced from the diagonal line along which all the other peptides were found. This "diagonal fingerprint" permitted us to isolate the single histidine peptide from the corresponding band of the preparative electrophoretogram. It was purified by electrophoresis at pH 1.9 and paper chromatography in a butanol—acetic acid—water (12:3:5, v/v/v). The peptide was identified on the basis of ninhydrin and Pauly reactions. The yield varied between 20 and 45%.

The amino-acid composition of these peptide was the following: lysine 1, histidine 1, methionine 2, glutamic acid 2, glycine 3 and valine 2.

The N-terminal of the peptide was histidine, as determined by the dansyl method. Accordingly, this peptide can be localized in the amino-acid sequence of actin described by Elzinga and Collins [17] between histidine-40 and lysine-50.

DISCUSSION

On partial carbethoxylation of G-actin the non-polymerizable fraction increases parallel with the modification of the four fast-reacting histidines.

The blocking of one of the histidine residues causes the loss of ability to polymerize in a fraction of about 25% of the total actin sample. As long as the four surface-exposed histidine residues are not fully carbethoxylated, there is a chance to obtain carbethoxylated but polymerizable fractions of actin. This suggests that the reactivity of the "active site" residue is about the same as that of the other accessible histidines, *i.e.* they react in a random fashion.

Thus the second-order rate constant of carbethoxylation can be calculated from the slope of the line (relative reactivity) of Fig. 1 and 2. This calculation allowed us to compare the reactivities of accessible histidine residues and α-N-acetylhistidine as a model compound.

The pseudo-first-order rate constant of the hydrolysis of diethylpyrocarbonate was found by Kivinen [18] to be $4.96 \times 10^{-5}\ s^{-1}$ at 0 °C. Taking this into account, the rate constants of carbethoxylation of the four accessible groups of G-actin and of the three accessible groups of F-actin were evaluated as $90\ M^{-1}\ s^{-1}$ and $112\ M^{-1}\ s^{-1}$, respectively.

Since under the same conditions a similar value, $96\ M^{-1}\ s^{-1}$ was obtained for α-N-acetyl-histidine, it

can be concluded that the examined histidine residues in G and F-actin are located on the molecular surface and their reaction is not influenced by steric factors.

The specificity of the reaction and the essential role of a single histidine residue are apparent from the fact that in the non-polymerizable fraction of actin isolated after treatment with [^{14}C]diethylpyrocarbonate only one histidine residue is blocked.

Moreover, unblocking of the modified histidine residues with hydroxylamine restored the ability to polymerize and the myosin ATPase activating effect (see Fig.4 and 5).

Three fast-reacting histidine residues were found in F-actin and four in G-actin. This difference in number, and our earlier observation [5] that the carbethoxylation of F-actin does not lead to depolymerization and it can be repolymerized, imply that the essential histidine residue becomes buried as a result of polymerization.

Similar buried residues have already been observed in actin: a cysteinyl group [19,—21] and some tyrosine and tryptophan residues [22]. These are probably buried as a result of monomer-monomer interaction.

As in our case histidine-40 is not carbethoxylated in F-actin, whereas its modification in G-actin prevents polymerization, it is likely to be located one of the contact surfaces. It should be noted, however, that an indirect effect *via* some structural change cannot be excluded.

The present work, in accord with the result of Ovádi and Keleti [23], shows that despite the lability of carbethoxy-histidine [6,24], the affected histidine can be identified in the primary structure of the modified proteins by diagonal electrophoresis under appropriate conditions. The selectivity of diethylpyrocarbonate for modification of histidine residues has been already observed [25—31]; now it has enabled us to localize the carbethoxy-histidine residues in the primary structure of the modified molecule and it seems therefore to be a promising tool for the study of the histidine residues in other proteins.

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