Interaction of Aldolase with Actin-Containing Filaments

STRUCTURAL STUDIES

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Electron micrographs of the paracrystals formed when fructose biphosphate aldolase (EC 4.1.2.13) is added to actin-containing filaments were analysed by computer methods so that ultrastructural changes could be correlated with the various stoichiometries of binding determined in the preceding paper [Walsh, Winzor, Clarke, Masters & Morton (1980) *Biochem. J.* 186, 89–98]. Paracrystals formed with aldolase and either F-actin or F-actin–tropomyosin have a single light transverse band every 38 nm, which is due to aldolase molecules cross-linking the filaments. In contrast, the paracrystals formed between aldolase and F-actin–tropomyosin–troponin filaments show two transverse bands every 38 nm: a major band, interpreted as aldolase binding to troponin, and a minor band, interpreted as aldolase cross-linking the filaments. The intensity of the minor band varies with Ca^{2+} concentration, being greatest when the Ca^{2+} concentration is low. A model for the different paracrystal structures which relates the various patterns and binding stoichiometries to structural changes in the actin-containing filaments is proposed.

In the preceding paper (Walsh *et al.*, 1980) evidence was presented on the stoichiometry of the binding of fructose biphosphate aldolase (EC 4.1.2.13) to actin-containing filaments derived from skeletal muscle. Filaments which contained only F-actin displayed an aldolase:actin binding ratio of approx. 1:14, and this ratio was increased to 2:14 in F-actin–tropomyosin filaments. When troponin was present in addition to F-actin and tropomyosin, an effect of Ca^{2+} on the aldolase binding was noted, with the binding ratio being 3:14 when the Ca^{2+} concentration was high (greater than 0.1 mM) and 4:14 when it was low (in the presence of EGTA). Clarke & Morton (1976) showed in previous electron-microscope studies that the binding of aldolase to these filaments is accompanied by the formation of paracrystalline bundles of filaments, which display characteristic cross-banding patterns. These supramolecular assemblies are of particular interest because there have been several reports of the presence of actin bundles *in situ* in a wide range of non-muscle cells of animal (Tilney, 1975) and plant (Palevitz & Hepler, 1975) origin. In addition, partially purified extracts from

brain (Bray & Thomas, 1976), *Physarum* (Spudich & Cooke, 1975), sea-urchin eggs (Kane, 1975) and *Limulus* acrosomes (Tilney, 1975) have yielded similar paracrystalline arrays under appropriate conditions.

In this paper, we present a structural analysis, based on electron microscopy and computer image processing, of the paracrystals formed with aldolase and F-actin, F-actin–tropomyosin and F-actin–tropomyosin–troponin. We also propose a structural model which correlates the various banding patterns with the observed stoichiometry of aldolase binding.

Methods and Materials

Proteins

Aldolase, F-actin, tropomyosin and troponin were prepared as described in detail in the preceding paper (Walsh *et al.*, 1979).

Electron microscopy

For examination by electron microscopy, aldolase and the various actin-containing filament preparations were mixed in 5 mM-imidazole/HCl/10 mM-KCl/1 mM- MgCl_2 /2 mM-dithiothreitol, pH 6.8 at 4°C, so

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that a final protein concentration in the range 0.25–0.5 mg/ml was obtained. When required, 0.1 mM- CaCl_2 or 0.2 mM-EGTA was also added. Paracrystals were formed by increasing the KCl concentration to 35 mM. For aldolase–F-actin and aldolase–F-actin–tropomyosin, paracrystals were also formed by the addition of BaCl_2 to a final concentration of 5 mM (Clarke & Morton, 1976). A drop of the required paracrystal suspension was applied to a carbon-coated grid and the excess was removed by touching with a filter paper. The preparation was then negatively stained with aq. 1% uranyl acetate as described by Huxley (1963). The grids were examined at either 60 or 80 kV in a Philips EM300 electron microscope and images were recorded on plates or sheet film at a magnification of approx. $\times 40000$. Magnifications were calibrated as described by Dickson (1974).

Computer image processing

To obtain projected axial density profiles, the micrographs were processed in a manner analogous to that described for the processing of paracrystals of tropomyosin (Stewart, 1975; Stewart & McLachlan, 1976). Areas were initially selected on the basis of straightness of the paracrystals and preservation (as assessed visually and by optical diffraction), and a raster scan was made with a Perkin Elmer PDS microdensitometer. The raster spacing was chosen to correspond to approx. 1 nm on the original paracrystal. These densities were stored on magnetic tape until required for processing. A projected density trace was produced by summing these densities in a direction perpendicular to the long axis of the paracrystal; bilinear interpolation was used when the raster scan was not aligned exactly with the paracrystal axis. Fourier transforms were computed by means of standard fast Fourier-transform algorithms, with a sampling frequency chosen to be large compared with the spacing of orders, so that errors owing to the reflections from the periodic structure not being located exactly on the sampling grid of the transform would be minimized (see Stewart & McLachlan, 1976). Projected traces and their transforms were then displayed graphically with the use of software supplied by the CSIRO Division of Computing Research and, when required, were recorded photographically on an ILL COMp80 computer-output microfilm device. The CSIRO CYBER 76 computer was used for all computations with software written in FORTRAN IV.

Optical diffraction

Optical diffraction patterns were observed with the apparatus described by Beaton & Filshie (1976).

Results

Plate 1 shows the different paracrystal banding patterns obtained when aldolase was mixed with F-actin, F-actin–tropomyosin and F-actin–tropomyosin–troponin in the presence and absence of Ca^{2+}

Fig. 1. Projected axial density traces of electron micrographs of paracrystals derived from actin-containing filaments (a) Magnesium paracrystal of F-actin–tropomyosin–troponin; (b) aldolase–F-actin paracrystal; (c) aldolase–F-actin–tropomyosin paracrystal; (d) aldolase–F-actin–tropomyosin–troponin paracrystal in the presence of 0.1 mM- CaCl_2 ; (e) as (d), but in 0.2 mM-EGTA instead of CaCl_2 . Bar represents 100 nm. The density units for each trace depend on a number of factors, such as the contrast of the original negative and the amount of background present owing to stain, support film etc. We have scaled the traces to compensate for these differences and to allow the axial periodicities to be clearly seen. There are also small variations in the linear scale from one trace to the next, owing to the well-known variability in the absolute magnification of the electron microscope because of lens hysteresis.

EXPLANATION OF PLATE I

Electron micrographs of paracrystals derived from actin-containing filaments

(a) Magnesium paracrystal of F-actin-tropomyosin-troponin; (b) aldolase-F-actin paracrystal; (c) aldolase-F-actin-tropomyosin paracrystal; (d) aldolase-F-actin-tropomyosin-troponin paracrystal in the presence of 0.1 mM- CaCl_2 ; (e) as (d), but in 0.2 mM-EGTA instead of CaCl_2 . Bar represents 100 nm.

ions. When troponin is present in the mixture, transverse bands are clearly visible on the paracrystals at 38 nm intervals. Clarke & Morton (1976) proposed that there may also be a very faint band between each major band. The pattern appears similar in the presence and absence of Ca^{2+} ions. Although there is some banding in the absence of troponin, it is somewhat weaker. For comparison, a magnesium paracrystal of F-actin-tropomyosin-troponin is also included (Plate 1a).

We first attempted to obtain a picture of the effect of filament composition and Ca^{2+} concentration on the distribution of aldolase in the paracrystals by producing axially projected density traces by densitometry of the different paracrystal images. This was done by densitometering the images on a raster and then summing the densities in a direction perpendicular to the long axis of the paracrystal. As the original pattern consisted of transverse bands, the effect of this summation was to enhance the signal-to-noise ratio. This effect is analogous to the well-known procedure of viewing such a pattern from the side, when the eye tends to perform a similar integration. We were further encouraged to use projected density traces by their effective use in the study of the transverse banding patterns in tropomyosin paracrystals (Stewart, 1975; Stewart & McLachlan, 1976). Typical projected axial density traces for the different paracrystals, and also, for comparison, of a magnesium paracrystal of F-actin-tropomyosin-troponin filaments, are shown in Fig. 1. These generally confirm the impression obtained by visual inspection of the original images. The axial density fluctuation is low in paracrystals lacking troponin and, when troponin is present, there is clearly one major band every 38 nm, with a minor band placed between each pair of major bands. The signal-to-noise enhancement obtained by this procedure has clearly been sufficient to establish the presence of the minor band. There is also a suggestion that the minor band is stronger in those paracrystals formed in the absence of Ca^{2+} (i.e. in the presence of EGTA), but the still substantial noise content of the traces makes it difficult to assess this point.

A more quantitative picture can be obtained by Fourier analysis. Essentially this technique involves decomposing the density trace into a sum of sine and cosine functions, and this allows a frequency spectrum to be obtained. In a periodic function, this frequency spectrum will have strong peaks corresponding to the period of the function and its overtones, which are generally referred to as 'orders'. However, the noise present in the image (in the present instance, probably due mainly to inhomogeneities in the carbon support film and stain and to defects in the paracrystals) is generally not periodic and its Fourier spectrum is approximately constant. The process of Fourier transformation therefore serves to effect a substantial

Fig. 2. Fourier spectra of projected axial density traces of electron micrographs of paracrystals derived from actin-containing filaments

(a) Magnesium paracrystal of F-actin-tropomyosin-troponin; (b) aldolase-F-actin paracrystal; (c) aldolase-F-actin-tropomyosin paracrystal; (d) aldolase-F-actin-tropomyosin-troponin paracrystal in the presence of 0.1 mM- CaCl_2 ; (e) as (d), but in 0.2 mM-EGTA instead of CaCl_2 .

separation of the periodic signal (in the present instance, owing to the banding pattern) from the noise, and this greatly facilitates an analysis of the signal.

Fig. 2 shows the Fourier spectra of the projected density traces shown in Fig. 1. With F-actin-tropomyosin-troponin magnesium paracrystals the axial periodicity is too weak to be detected, and no clear peaks emerge in the Fourier spectrum. The periodicity is a little stronger in the F-actin-aldolase and F-actin-tropomyosin-aldolase paracrystals, and here the first order is quite clear. The absence of higher orders means that, at least to the resolution of the present

Fig. 3. Illustration of the contribution of the first and second orders to the projected axial density traces of the paracrystals which contain troponin (five 38 nm repeats are shown) (a) Density caused by the first order; (b) density caused by the second order; (c) combined density. The first order only contributes to the density of the major band, whereas the second order contributes to both bands. Bar represents 100 nm.

study, the banding pattern in these paracrystals can be represented as a single cosine wave. This means that the density corresponding to the aldolase tends to be spread out to form a fairly broad band. This correlates well with the original micrographs (Plate 1), in which the band can be clearly seen to be diffuse in character. The Fourier spectra of the paracrystals that contain troponin both have very clear first and second orders (see Fig. 2), both having a phase angle of 0 when referred to the centre of the major band as origin.

The Fourier spectra can be simply related to the banding patterns in the paracrystals. As illustrated in Fig. 3, when the waves corresponding to these two orders are added, a pattern containing one major and one minor band is produced. However, the first order contributes only to the density of the major

band, whereas the second order contributes to both. The relative amplitudes of these two orders therefore can be used to give a measure of the relative densities of the major and minor bands. If the first order is strong relative to the second, then the minor band will be weak, or alternatively, if the second order is strong, then the minor band will approach the density of the major band (in the limiting case, when these bands are of equal density, the first order becomes zero). It is clear from Fig. 2 that, in the presence of Ca^{2+} ions, the amplitude of the second order decreases relative to that of the first. This implies that, in the absence of Ca^{2+} ions, the density of the minor band increases relative to that of the major band, which confirms the impression gained from the projected density traces. Unfortunately, it is not possible to determine accurately a baseline for these traces, and so the exact magnitude of the change in density of the minor band cannot be assessed.

The results in the preceding paper (Walsh *et al.*, 1980) have shown that an increased binding of aldolase to F-actin-tropomyosin-troponin filaments also occurs in the presence of 0.2 mM-EDTA, and so the Fourier spectra imply that this additional binding is taking place at the site giving rise to the minor band in the electron micrographs of the negatively stained paracrystals.

Discussion

(a) Structural interpretations

On the basis of the results obtained from both the binding studies and the analysis of the electron micrographs, we are able to propose a model for the structural interactions involved in the range of paracrystals formed with aldolase. Although this model is consistent with all the data available to date, it should be considered as tentative until confirmation is obtained from structural studies of higher resolutions, particularly with respect to the three-dimensional organization of the filaments.

(i) *F-actin-aldolase paracrystals*. The stoichiometry of binding in this system was observed to be approx. 1 aldolase molecule per 14 G-actin units (Walsh *et al.*, 1980). As all the G-actin units are usually considered to be equivalent in the F-actin structure, one must initially question why only a small proportion of them bind to aldolase. Why doesn't each actin subunit bind to aldolase?

We propose that the 1:14 stoichiometry is produced by aldolase only binding to actin when it is able to attach to two G-actin units simultaneously, and that this can only occur when aldolase forms a bridge between two adjacent filaments. As the actin filaments have a helical structure, one would expect that a positioning of G-actin units favourable to such an interaction would occur twice in the helix pitch

length (since it is a two-stranded helix), that is at approximately every 38 nm. This would correspond to about once every 7 actin units. However, as each aldolase molecule would be binding to two G-actin units, only 1 aldolase molecule per 14 actin units would be required. Furthermore, one would expect that, because two binding sites would be involved per aldolase molecule, this method of binding would be considerably stronger than one involving a single contact between actin and aldolase. We are further encouraged in this interpretation by studies on actin-containing arrays from sea-urchin eggs (De Rosier *et al.*, 1977), which showed an analogous form of binding between a cross-linking protein and actin.

In practice, there are two complications to this model. The first is that the paracrystals appear to be three-dimensional bundles, and the second is that they generally only contain a small number of filaments. The effect of the first would be to require a higher ratio of aldolase to actin to encompass three-dimensional binding because there would be more chances for favourable positioning of actin units as the number of nearest-neighbour filaments increases. If the filaments were only to pack into sheets, then there would only be two nearest neighbours, but, depending on the mode of packing in a three-dimensional arrangement, there could be as many as six nearest neighbours, which could entail as many as three times as many aldolase molecules, although this number could be decreased somewhat if there were strict stereochemical constraints placed on the geometry of binding: see De Rosier *et al.* (1977) for a detailed discussion of this point. The effect of the small number of filaments in each bundle, however, would be to decrease this ratio, because many of the eligible actin units on the outermost filaments would not be able to form cross-links with adjacent filaments. The two effects would therefore tend to cancel out, and so we conclude that the observed stoichiometry is in reasonable accord with the model.

(ii) *F-actin-tropomyosin-aldolase paracrystals*. We propose that the structure of these paracrystals is closely related to those formed between actin and aldolase only, except that the tropomyosin allows the binding of additional aldolase. The most likely manner in which this could occur is for a specific binding between the tropomyosin and the enzyme, although we consider it highly likely that the presence of actin is also required for the interaction to proceed (see Walsh *et al.*, 1980). This interaction possibly gives rise to additional cross-links between adjacent filaments. Because the tropomyosin follows a helical path of the same pitch as that of the actin, we would expect these cross-links to be formed with the same frequency as those between G-actin units alone, and this would account for the doubling of the stoichiometry of aldolase binding from 1:14 to 2:14. However, because the actin and tropomyosin helices are

not in phase (that is to say, the helical paths followed by tropomyosin lie between the actin helices), we would expect that the binding sites for each type of cross-link would occur at slightly different axial positions along each filament. However, at the resolution of this study, the density from these two sites would merge to produce a single band, as seen in Plate 1 and Fig. 1.

(iii) *F-actin-tropomyosin-troponin-aldolase paracrystals*. The stoichiometry observed in these paracrystals is either 3:14 or 4:14, depending on the Ca^{2+} concentration. We suggest that this binding is made up of two components. The first is a specific 1:1 binding of aldolase to troponin (Walsh *et al.*, 1980), which does not contribute to the formation of cross-bridges between the filaments in the paracrystals. As the stoichiometry of troponin to actin is 1:7 (Weber & Murray, 1972), the specific aldolase-troponin binding would account for 2 aldolase molecules per 14 actin units, which would leave respectively 1 and 2 aldolase molecules per 14 actin units unaccounted for in the aggregates formed in the presence and absence of Ca^{2+} ions. We propose that these molecules form cross-bridges between the filaments in a manner analogous to that proposed for the paracrystals formed in the absence of troponin.

The effect of Ca^{2+} concentration can be explained by the structural changes that it produces in the actin-tropomyosin-troponin filaments. The major change brought about by Ca^{2+} ions in this system is thought to involve the movement of tropomyosin in the groove of the actin helix, which may be accompanied by a small change in the helical pitch of the actin (Gillis & O'Brien, 1976). When the concentration of Ca^{2+} is high, the tropomyosin is located deep in the actin groove; when the Ca^{2+} concentration is low, the tropomyosin is moved out of the groove by a conformational change in the troponin (Haselgrove, 1972; Huxley, 1972; Parry & Squire, 1973; Wakabayashi *et al.*, 1975). The binding studies showed that the addition of tropomyosin to F-actin resulted in an increase in the aldolase-binding stoichiometry from 1:14 to 2:14, the same change observed in paracrystals formed in the presence and absence of Ca^{2+} ions. It is suggested that the increased aldolase binding caused by tropomyosin is only effective when this molecule occupies a position out of the actin groove. When troponin is present, tropomyosin only occupies this position in the absence of Ca^{2+} ions. When the tropomyosin is moved into the groove, its ability to bind further aldolase would be lost, either because the tropomyosin has been rotated (Stewart & McLachlan, 1976; Parry, 1976), or because its changed position relative to the G-actin subunits has destroyed the stereochemical relationship required to form a G-actin-tropomyosin-aldolase complex. This hypothesis is also consistent with the binding studies (Walsh *et al.*, 1980), which

indicated that the binding of aldolase to troponin and tropomyosin in the absence of actin did not show any effect of Ca^{2+} .

(b) *Biochemical implications*

The binding of a number of glycolytic enzymes, including aldolase, to the I-band of muscle (Sigel & Pette, 1969) points to a functional role *in vivo*. However, the organization of the actin filaments in muscle is such that the filament spacing would probably preclude the cross-linking of filaments by aldolase. Interactions of aldolase molecules with troponin and with the secondary binding site not involving cross-linking could account for the localization observed by Sigel & Pette (1969). The significance of this association for enzyme function is discussed in the preceding paper (Walsh *et al.*, 1980). The formation of these paracrystalline arrays is not limited to actin filaments with aldolase. They are also produced by the regulatory enzymes phosphofructokinase, glyceraldehyde 3-phosphate dehydrogenase and pyruvate kinase (D. J. Morton & F. M. Clarke, unpublished work).

The biological role of actin-enzyme bundle formation may conceivably be found in non-muscle cells, where the presence of actin in the form of bundles or stress fibres has already been demonstrated (Lazarides, 1975; Lazarides & Weber, 1974). If indeed it can be demonstrated that non-muscle actin interacts with glycolytic enzymes in the manner shown here for muscle actin, then glycolytic enzymes such as aldolase which bind to actin could exert a regulatory role in non-muscle-cell motile functions. One can note in this connection the similarity in properties of muscle and non-muscle actin (Gordon *et al.*, 1977), as well as the obligatory involvement of glycolysis in motile functions such as phagocytosis (Sbarra & Karnovsky, 1960).

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References

- Beaton, C. D. & Filshie, B. (1976) *J. Gen. Virol.* **31**, 151–161
- Bray, D. & Thomas, C. (1976) *J. Mol. Biol.* **105**, 527–544
- Clarke, F. M. & Morton, D. J. (1976) *Biochem. J.* **159**, 797–798
- De Rosier, D., Mandelkow, E., Silliman, A., Tilney, L. & Kane, R. (1977) *J. Mol. Biol.* **113**, 679–695
- Dickson, M. R. (1974) *Lab. Pract.* **23**, 392–395
- Gillis, J. M. & O'Brien, E. J. (1976) *J. Mol. Biol.* **99**, 445–459
- Gordon, D. J., Boyer, J. L. & Korn, E. D. (1977) *J. Biol. Chem.* **252**, 8300–8309
- Haselgrove, J. C. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 341–351
- Huxley, H. E. (1963) *J. Mol. Biol.* **7**, 281–308
- Huxley, H. E. (1972) *Cold Spring Harbor Symp. Quant. Biol.* **37**, 361–376
- Kane, R. E. (1975) *J. Cell Biol.* **66**, 305–315
- Lazarides, E. (1975) *J. Histochem. Cytochem.* **23**, 507–552
- Lazarides, E. & Weber, K. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2268–2272
- Palevitz, B. A. & Hepler, P. K. (1975) *Planta* **125**, 261–271
- Parry, D. A. D. (1976) *Biochem. Biophys. Res. Commun.* **68**, 323–328
- Parry, D. A. D. & Squire, M. J. (1973) *J. Mol. Biol.* **75**, 33–55
- Sbarra, A. J. & Karnovsky, M. L. (1960) *J. Biol. Chem.* **234**, 1355–1362
- Sigel, P. & Pette, D. (1969) *J. Histochem. Cytochem.* **17**, 225–237
- Spudich, J. A. & Cooke, R. (1975) *J. Biol. Chem.* **250**, 7485–7491
- Stewart, M. (1975) *Proc. R. Soc. London, Ser. B* **190**, 257–266
- Stewart, M. & McLachlan, A. D. (1976) *J. Mol. Biol.* **103**, 251–269
- Tilney, L. (1975) *J. Cell Biol.* **64**, 289–310
- Wakabayashi, T., Huxley, H. E., Amos, L. A. & Klug, A. (1975) *J. Mol. Biol.* **93**, 477–497
- Walsh, T. P., Winzor, D. J., Clarke, F. M., Masters, C. J. & Morton, D. J. (1980) *Biochem. J.* **186**, 89–98
- Weber, A. & Murray, J. M. (1972) *Physiol. Rev.* **53**, 613–673