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Subunit Interaction in Native and Modified Muscle Phosphorylases*

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ABSTRACT: It is shown by sedimentation and gel electrophoresis that both muscle phosphorylases *a* and *b* exist under normal circumstances in equilibrium between dimeric and tetrameric forms. The equilibrium is displaced in favor of the tetramer by the addition of 5′-adenylic acid. The nature of the sedimenting boundaries indicates that the association equilibrium is not in general a rapid process, and may thus involve a conformational change in the protein. Reaction with *p*-mercuribenzoic acid (PMB) is known to lead to monomers from either phosphorylase; these monomers are still found to interact with adenylic acid, the phosphorylase *b* giving predominantly dimer and some polydisperse aggregates, phosphorylase *a* only large aggregates. On lowering the ionic strength, the PMB monomers of phosphorylase *b* give place to dimer, whereas phosphorylase *a* produces a remarkable series of polymers, which are shown to include odd species, notably the trimer. Nine bands are resolved by gel electrophoresis, and the first four are resolved in the analytical ultracentrifuge. In the electron microscope native dimeric phosphorylase *b* is shown to consist of two indistinguishable, roughly ellipsoidal subunits, and the phosphorylase *a* and tetrameric form of phosphorylase *b*, of four such subunits, arranged with their centers at the corners of a rhombus. Each subunit corresponds to a monomer of 92,500 mol wt. Examination of phosphorylase *a* from crystals immediately after dissolving in a medium of low cysteine content shows polymers consisting apparently of dimers assembled with their long axes parallel. It is concluded that certain lattice contacts can become association sites in the dispersed state. The PMB aggregates of phosphorylase *a* take the form of clusters of monomers, which indicates that cyclic rather than linear aggregation is favored. By all available criteria the PMB monomers are intact, and not unfolded, protein; arguments are given for the presence of at least three different binding sites in each phosphorylase monomer, even in the native state, two of which are weakened when PMB is bound. Application of the hydrodynamic theory of Kirkwood for assemblages of monomeric beads leads to calculated sedimentation coefficients and frictional ratios which agree remarkably with experimental data, and are more compatible with cyclic than with linear structures.

The native muscle phosphorylases exist in two aggregation states, a dimer (Keller and Cori, 1953; Keller, 1955) which has now been found (Seery *et al.*, 1967) to have a molecular weight of 185,000, and a tetramer of mol wt 370,000. Phosphorylase *a* differs from *b* in having one seryl residue in each subunit in the form of a phosphate ester. Phosphorylase *a* is normally tetrameric, but is capable of dissociating at high ionic strength into a still active dimer (Wang and Graves, 1963). Phosphorylase *b*, in the absence of 5′-adenylic acid (AMP)[1], is a dimer, which on activation with AMP is converted into the active tetrameric form (Kent *et al.*, 1958). As with other such systems, it is therefore evident that the function of the enzyme is intimately connected with its aggregation state. When phosphorylase *a* is treated with organomercurials, such as *p*-mercuribenzoic acid (PMB), the enzyme is reversibly inactivated and the monomer (which is not necessarily however a single polypeptide chain) is generated (Madsen and Cori, 1956). The same authors also reported that these monomers slowly associate to form very large aggregates. Slow aggregation has likewise been observed in the native protein when no protective sulfhydryl compounds are present (Krebs and Fischer, 1962). We have observed that under well-defined conditions of ionic strength the PMB-treated monomer gives rise to a series of discrete polymers. We report here a study of the nature of the polymerization process, and the relation

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[1] Abbreviations used: PMB, *p*-mercuribenzoic acid; AMP, 5′-adenylic acid.

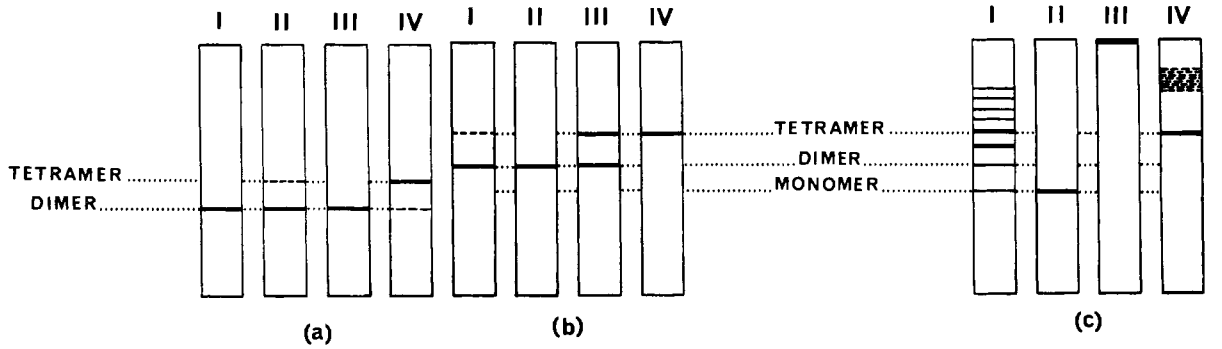

FIGURE 1: Polyacrylamide gel electrophoresis of phosphorylases *a* and *b*. (a) Gel buffer: 0.11 M glycine–Tris (pH 7.2). Gels were preequilibrated with 0.01 M β-mercaptoethanol. (b) Gel buffer: standard Tris–glycine system (pH 8.75) (see text). (c) Gel buffer: as in b but with 5×10^{-3} M PMB in gel and reservoir buffers. Four different samples were run in each gel system: (I) phosphorylase *a*, (II) phosphorylase *b*, (III) phosphorylase *a* with AMP (5×10^{-3} M), and (IV) phosphorylase *b* with AMP (5×10^{-3} M).

between these aggregates and the native states of the protein.

Experimental Section

Phosphorylase *b* was prepared from rabbit skeletal muscle according to Fischer and Krebs (1958); the protein was stored as a crystalline suspension in the presence of 0.001 M AMP and 0.03 M cysteine at pH 6.8. For removal of the AMP the protein was passed through a charcoal-cellulose column, as also indicated by Fischer and Krebs. Phosphorylase *a* was obtained from Sigma Chemical Co. and from Worthington Biochemical Corp. Both phosphorylases under standard conditions gave single sharp boundaries in the ultracentrifuge and single zones in polyacrylamide gel electrophoresis. Adenylic acid was obtained from Sigma Chemical Co., and PMB from British Drug Houses Ltd.

Polyacrylamide gel electrophoresis was performed in an apparatus similar to that described by Davis (1964) and Clarke (1964) at room temperature, using a fan to cool the gels. The standard (optimal) acrylamide concentration was 3.5%, with a ratio of acrylamide to cross-linking agent (methylenebisacrylamide) of 20:1. The medium for both aggregation and electrophoresis was a Tris–glycine buffer (5.5×10^{-2} M glycine and 5.8×10^{-3} M Tris, pH 8.75). Samples were layered on the gel in 5% sucrose, and bromophenol blue was used in one tube as a marker. Electrophoresis was continued for twice the time required for the dye to leave the gel. The reservoir buffer was replaced once in the middle of each run to prevent contamination with electrode products. The gels were stained with Amido Black and destained electrophoretically. Excess PMB formed a white opacity in the gel, which was dissolved by soaking the gels in 0.1 M cysteine hydrochloride solution. The cleared gels were evaluated with a Joyce-Loebl microdensitometer.

Sedimentation measurements were made in a Spinco Model E ultracentrifuge using the schlieren optical system and 12-mm 4° sector centerpieces. The same buffer system was again used; the temperature was maintained at 20°, and the standard speed was 59,780 rpm. Protein concentrations were determined spectrophotometrically using a specific absorptivity (278 mμ) of $E^{1\%}_{1\,cm}$ 11.9 (Appleman *et al.*, 1963).

Electron Microscopy. Samples diluted to have a fixed protein concentration of about 0.1 mg/ml were negatively stained as previously described (Valentine *et al.*, 1966) and examined in a Philips EM 200 instrument. For electron microscopy the phosphorylase *b* was dialyzed overnight against 0.04 M sodium glycerophosphate -10^{-3} M dithiothreitol (pH 7.0). The phosphorylase *a* was kept as a suspension in 0.0015 M EDTA, 0.002 M sodium glycerophosphate, and 0.1 M sodium fluoride (pH 6.6). Before use, the sample was dissolved by addition of neutralized cysteine to a concentration of 0.01 M, 0.05 M phosphate buffer (pH 7.0) was added as diluent, and the grid was prepared immediately. Several preparations were also pretreated with glutaraldehyde as a cross-linking agent before placing on the grid. For the examination of phosphorylase *a* aggregates, PMB in the Tris–glycine buffer was added to give 10^{-3} M PMB, shortly before preparation of the grids.

Results

Native Proteins. Native phosphorylase *a* has been reported to have a sedimentation coefficient of 13.2 S (Keller and Cori, 1953). We find that in 0.02 M sodium glycerophosphate and 0.03 M cysteine a single symmetrical boundary is observed only when the protein concentration is about 3 mg/ml or greater (at 20°) in our buffer system. As the protein concentration is lowered, a second and slower component progressively appears, which evidently corresponds to the dissociated (dimeric) form of the enzyme. This component is partly resolved, and since a rapid dimerization equilibrium can lead only to a single asymmetric boundary (Gilbert, 1955; Gilbert and Jenkins, 1959), it appears that we are dealing here with a relatively slow dissociation. In gel electrophoresis in the absence of AMP (Figure 1b) the dimer is overwhelmingly favored, the migration rate being identical with that of phosphorylase *b*.

When AMP is introduced into the buffer, the phosphorylase *b* is wholly converted into a tetramer, whereas phosphorylase *a* migrates as a mixture of tetramer and dimer. The presence of the two zones again indicates a

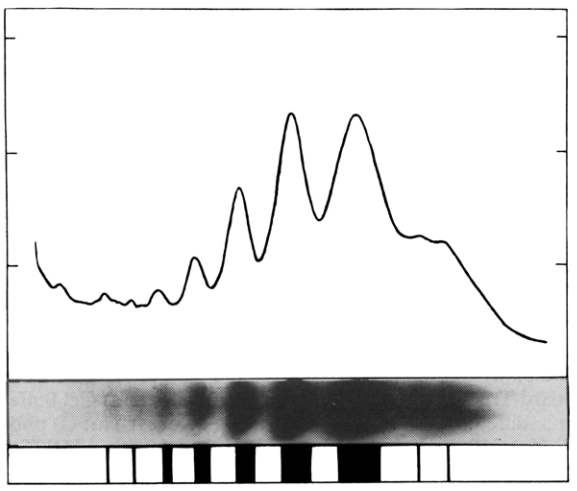

FIGURE 2: Polyacrylamide gel electrophoresis of polymers formed by addition of PMB to phosphorylase *a* at low ionic strength and corresponding densitometer trace.

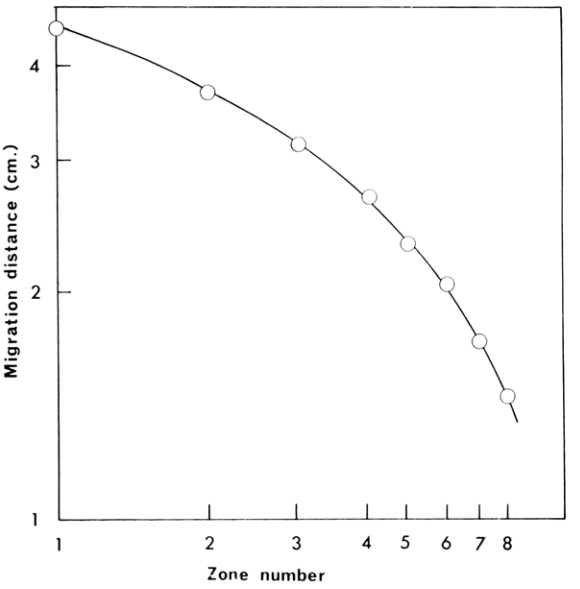

FIGURE 3: Logarithmic plot of zone number against migration distance for polymers after electrophoresis in polyacrylamide gel.

slow or "frozen" equilibrium. At pH 7.2 and in the presence of β-mercaptoethanol (Figure 1a) the phosphorylase *a* travels entirely as the dimer, even in the presence of AMP. A direct quantitative comparison of the electrophoretic and sedimentation results is not possible, since the concentration within the migrating electrophoretic zone is unknown, and the temperature inside the electrophoresis gel during the experiment is relatively high (probably above 30°).

Action of PMB. When PMB is added to phosphorylase *b*, electrophoresis in Tris–glycine buffer (Figure 1c) reveals only a zone migrating ahead of the dimer position, and corresponding to the monomer of 5.6 S. When AMP is added the monomer vanishes and gives place to dimer, as well as a smear of polydisperse aggregated material. In the ultracentrifuge in the same solvent the aggregate appears as a rather broad boundary, separated from the more prominent dimer boundary, and sedimenting at about 17 S.

With phosphorylase *a* however a new phenomenon is observed: at relatively high salt concentration (0.1 M KCl) sedimentation shows only the monomer of 5.6 S (*cf.* Madsen and Cori, 1956). In our standard Tris–glycine system, on the other hand, we observe a remarkable series of zones in gel electrophoresis, the first two of which occupy the positions associated with the monomer and dimer. Addition of AMP leads to the formation of large aggregates which do not enter the electrophoretic gel, and may precipitate. Figure 2 shows a typical gel pattern together with densitometer trace. A comparison of such gels with those of Figure 1 shows that the third band from the anodic end lies between the dimer and tetramer positions and is evidently a trimer. In Figure 3 we show a logarithmic plot of migration against DP, after Smithies and Connell (1959), and it is seen that the components form a smooth progression, again indicating that they are successive oligomers, with no missing components.

In the ultracentrifuge under the same conditions, the first four components are well resolved (Figure 4a). Corrections were applied for the Johnston–Ogston effect to give $s^0_{20,w}$ values for the four components. Their relative proportions were obtained from the areas under the schlieren boundaries, corrected only for radial dilution. The sedimentation coefficient for the first boundary is 5.9 S, and it therefore evidently represents the monomer. The second peak sediments at 8.2 S, which corresponds to the dimer (*e.g.*, phosphorylase *b*). The third is 11.5 S and the fourth 13.2 S, which corresponds to the tetramer, as in native phosphorylase *a* (Keller and Cori, 1953; Keller, 1955). These results are summarized in Table I.

The resolution of these boundaries again indicates that under these conditions there is no rapid polymerization equilibrium. Dilution of the solution does not give rise to any major redistribution of the schlieren areas. When the solution is left to stand overnight most of the protein then sediments as a broad boundary of about 12.2 S with a small 7S component. This state appears as judged by the electron microscopy (see below) to represent denatured protein. Addition of AMP to the PMB-treated protein leads to immediate precipitation.

TABLE I: Sedimentation Coefficients and Relative Concentrations of PMB Polymers of Phosphorylase *a* Resolved in the Ultracentrifuge.

Component	1	2	3	4
Sedimentation coefficient (S)	5.9	8.2	11.5	13.2
Relative concentrations from a typical polymerization experiment (%)	35	22	30	14

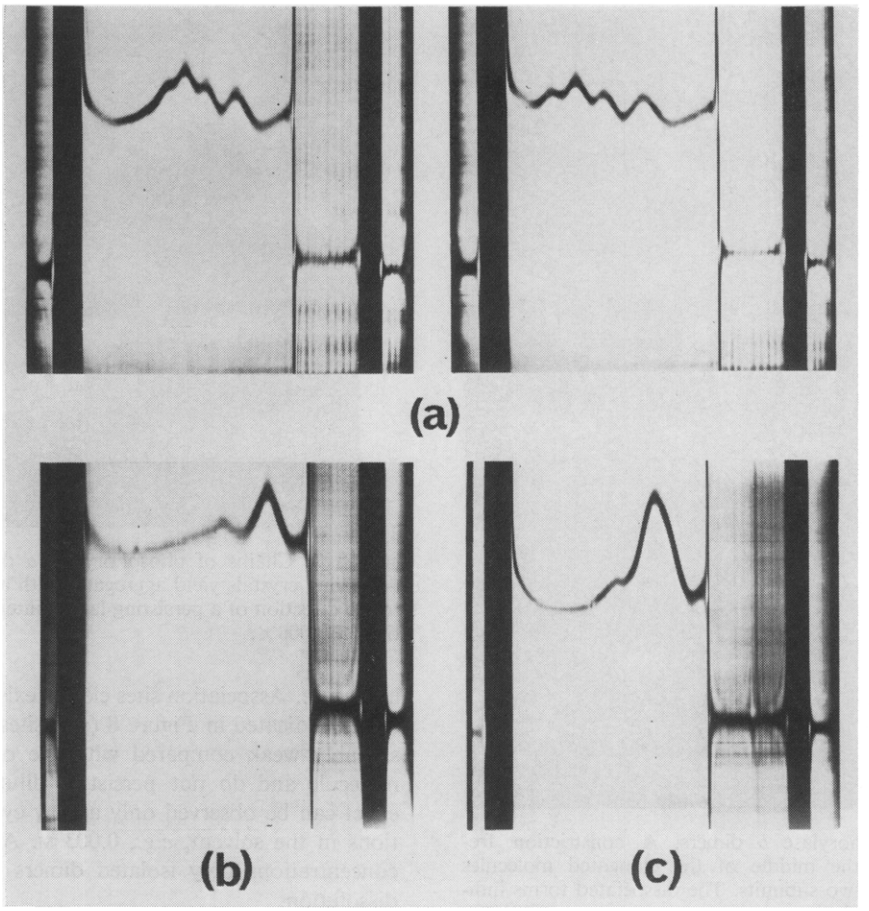

FIGURE 4: Sedimentation of phosphorylase after treatment with PMB (5×10^{-3} M). (a) Phosphorylase *a* in Tris–glycine buffer (pH 8.75). Schlieren phase–plate angle 40°. Exposures at 8 (left) and 16 min (right) after reaching speed. (b) Phosphorylase *b* under the same conditions as a. Schlieren phase-plate angle 30°. Exposure at 8 min after reaching speed. (c) Phosphorylase *b* under the same conditions as a with 10^{-3} M AMP. Schlieren phase-plate angle 40°. Exposures at 8 min after reaching speed. Sedimentation in all pictures is from right to left.

Phosphorylase *b* treated with PMB in the same buffer produces two sedimenting boundaries. At a protein concentration of 8 mg/ml, their sedimentation coefficients are 6.2 and 12.5 S, which may be monomer and denatured material (Figure 4b). On dilution the equilibrium shifts and the relative proportions of the components change. Applying the concentration dependence of the sedimentation coefficient of the monomer under comparable conditions, one obtains a $s^0_{20,w}$ value of 5.7 S. Addition of AMP leads to some increase in aggregation, with components of 8.6 and 17.3 S (Figure 4c).

Electron Microscopy. ISOLATED MOLECULES. Native phosphorylase *b* shows elongated molecules with a constriction at the middle suggesting that they consist of two particles (Figure 5). The molecules may be identified as the dimers by their dimensions, which measurements of the length and variable width indicate to be about $110 \times 65 \times 55$ Å. A probable lower limit for the volume will be obtained by regarding the molecule as an ellipsoid with axes of these lengths, and an upper limit by taking it to be cylindrical. With the partial specific volume of 0.74, the two models correspond to molecular weights of 165,000 and 250,000, respectively, which straddle the actual molecular weight of 185,000 for phosphorylase *b* dimers. In the electron microscope the tetrameric forms of phosphorylases *a* and *b* are indistinguishable and have a characteristic rhombic profile (Figure 6). The mean length of each side measures 110 Å. The suggested arrangement of the subunits is indicated schematically in Figure 8.

CRYSTALLINE STATE. In electron micrographs of phosphorylase *b* crystals the following periodicities are observed (Valentine and Chignell, 1968): $a = c = 120$ Å, $\beta = 107°$, which is taken to define the angle of the rhombus (above), and in a perpendicular plane, $a = 120$ Å, $b = 95$ Å, $\gamma = 90°$. From determinations of crystal density and the partial specific volume, one obtains a molecular weight for the tetramer of 365,000 which agrees closely with the hydrodynamic value of Seery *et al.* (1967).

AGGREGATED STATE. Figure 7 shows an electron micrograph of phosphorylase *a*, fixed immediately after dispersal of the crystals in the solvent. The crystals are slow to dissolve, and one sees here an intermediate state in the solution process, in which at least one of the lattice interactions still persists. The long strings of molecules are in effect *dimers* of phosphorylase *a*, lying parallel, so that a repeat distance of some 70 Å is observed, which corresponds to the long axes of the monomer units, oriented parallel to the length of the

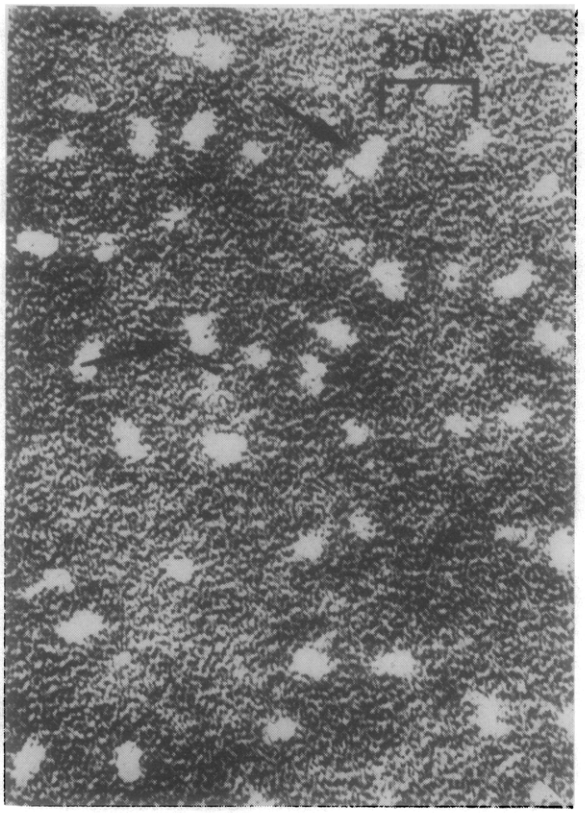

FIGURE 5: Phosphorylase *b* dimers. A constriction frequently seen at the middle of the elongated molecules (arrows) suggests two subunits. The dissociated forms indidate a nearly spherical monomer; magnification, 500,000×.

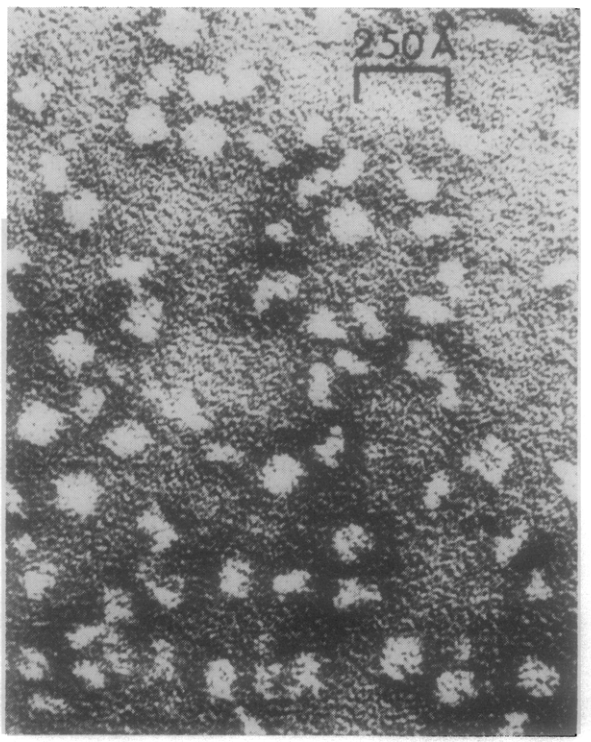

FIGURE 6: Phosphorylase tetramers. The tetrameric forms of phosphorylases *a* and *b* are indistinguishable and show a rhombic profile. The preparation also contained dimers; magnification, 500,000×.

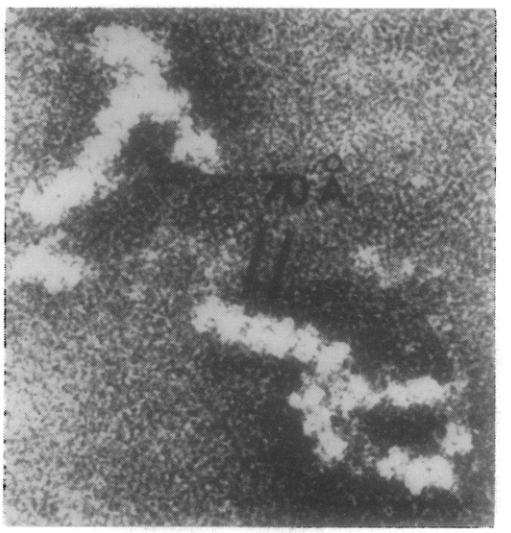

FIGURE 7: Chains of phosphorylase *a* dimers. The slowly dissolving crystals yield aggregates with a clear 70 Å repeat in the direction of a persisting lattice interaction; magnification, 500,000×.

aggregate. Association sites clearly exist therefore in the region indicated in Figure 8 ($\gamma\gamma'$ sites), which are presumably weak compared with the other sites of the molecule and do not persist in dilute solution. This effect can be observed only at low cysteine concentrations in the solvent, *e.g.*, 0.003 M. At higher cysteine concentrations only isolated dimers are observed on dissolution.

Figure 9 shows the aggregates formed by phosphorylase *a* under the action of PMB and the conditions described above. The polymers in this case are not linear arrays, and clusters of trimers, tetramers, etc., can be distinguished. It appears therefore that closed systems are preferred under these circumstances. After standing overnight, blurred clusters of presumably denatured protein are found.

Discussion

It has been shown that both phosphorylases *a* and *b* exist in the form of the dimer 185,000, the tetramer, or a mixture of the two, according to the concentration of the protein. The equilibrium is displaced in favor of the tetramer by the allosteric activator, AMP. It is curious that the dimerization process cannot be regarded in terms of a simple association–dissociation equilibrium, since the reaction is more or less slow. This suggests that at least under some circumstances the aggregation state is determined by conformational changes in the protein.

Dimeric phosphorylase *b* is shown in the electron microscope to consist of two indistinguishable roughly ellipsoidal subunits. Tetrameric phosphorylase *a* may be envisaged as being formed by the association of two such molecules and is a cyclic structure. It has not been established whether the subunits in the phosphorylases are identical or different (Krebs and Fischer, 1962). Certainly each one carries an active site and an AMP binding site. In either case the tetrameric structure

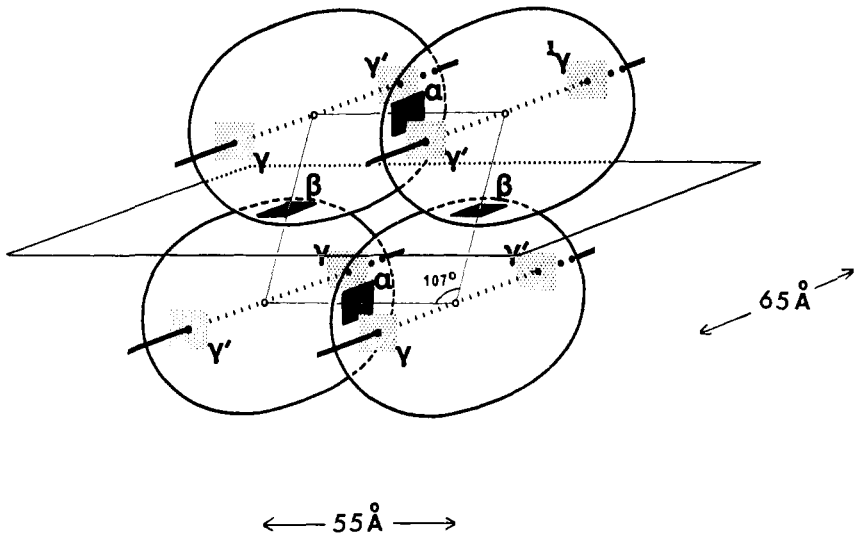

FIGURE 8: Subunits and binding sites of muscle phosphorylases (schematic).

TABLE II: Calculated and Observed Frictional Properties of Phosphorylase Polymers.[a]

Configuration	s_{calcd}	s_{obsd}	s_{obsd}: Native	f_{calcd}: × 10^8	$(f/f_0)_{calcd}$	$(f/f_0)_{obsd}$
○		5.6				1.28
⁞	8.4	8.2	8.4	9.61	1.35	1.35
⁞	10.3			11.80	1.26	
		11.5				1.29
△	11.2			10.82	1.33	
⁞	11.7			13.84	1.54	
⁞		13.2				
□	13.3		13.5	12.18	1.36	1.35
□□□	20.0	20[b]		16.18	1.43	1.43

[a] The values of $(f/f_0)_{obsd}$ in the last column are calculated from the corresponding s_{obsd}(S) on the same line, taking molecular weights as multiples of the monomer weight of 92,500. [b] Cold aggregates of phosphorylase *b* (Graves *et al.*, 1965) and aggregates by removal of thiol from phosphorylase *a* (Seery *et al.*, 1967).

which we observe (D_2 or C_2 symmetry, according to whether the subunits are identical or of two different types) conforms with the symmetry requirements proposed by Monod *et al.* (1965) for an allosteric system. No evidence is found in the electron microscope of any further subunit structure within each monomer.

The polymerization of the PMB-treated monomer is again a slow process. The sedimentation as well as gel electrophoresis results show that odd as well as even polymers are formed (most notably the trimer). Since the aggregation starts from the monomer, such species are not necessarily unexpected, especially since it must be supposed that the PMB blocks or grossly weakens the $\alpha\alpha'$ and weakens the $\beta\beta'$ association (Figure 8). For a strictly linear aggregation process, the relative concentrations of the oligomers should follow the standard expressions for distribution of DP's (Stockmayer, 1943; Mark and Tobolsky, 1950). For observed proportions of unpolymerized monomer, theoretical distributions were calculated for di-, tri-, and tetrafunctional polymerizations, and in no case was it possible to simulate the relative proportions of oligomers in fact obtained from the areas under the schlieren boundaries in such experiments as that of Figure 4. The most indicated

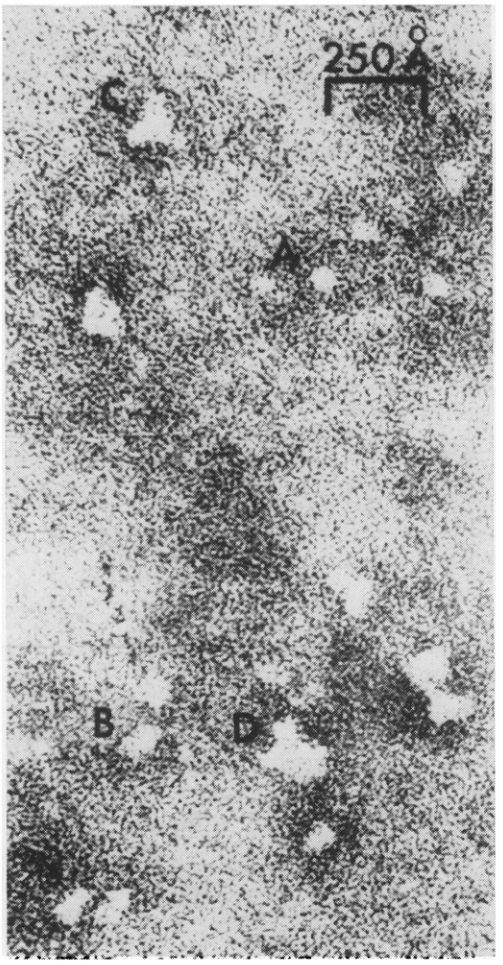

FIGURE 9: Polymers formed by PMB-treated monomers. The monomer (A), dimer (B), trimer (C), and higher polymer (D) show cyclic rather than linear polymerization; magnification, 500,000×.

explanation is in terms of cyclic rather than linear aggregation, and the large proportion of trimer supports this surmise.

We now attempt to rationalize the measured frictional properties of the oligomers. Beginning with literature data summarized by Krebs and Fischer (1962) for the PMB monomer, *viz.*, sedimentation coefficient 5.6 S, molecular weight 92,500 (Seery *et al.*, 1967), and partial specific volume 0.737, the frictional coefficient (ζ) is found to be 5.66×10^{-8}, and the frictional ratio 1.27.

According to Kirkwood (1954; see also Bloomfield *et al.*, 1967), for an assemblage of linked particles, the frictional coefficient is given by

$$= n\zeta\left[1 + (\zeta/6\pi\eta_0 n)\sum_{l=1}^{n}\sum_{\substack{s=1 \\ l\neq s}}^{n}\langle r_{ls}^{-1}\rangle\right]^{-1}$$

where n is the number of monomers, ζ the frictional coefficient of the monomer, η_0 the solvent viscosity, and $\langle r_{ls}^{-1}\rangle$ the mean reciprocal center-to-center distance between the subunits. Taking the experimental value of ζ derived above, and the corresponding values of $\langle r_{ls}\rangle^{-1}$ for linear and cyclic assemblies of ellipsoids with center-to center distances determined by electron microscopy on the native dimer and tetramer, we obtain the calculated sedimentation coefficients given in Table II. There is remarkable agreement with experimental data, which may indeed be partly fortuitous. If however the validity of the calculation be accepted, the results clearly show that both the trimer and tetramer are cyclic. This is consistent with the absence of linear structures in the electron micrographs. It may be pointed out that identical values for the frictional ratios of phosphorylases *a* and *b*, at first sight unexpected (see, *e.g.*, Krebs and Fischer, 1962), are in fact consistent with the demands of Kirkwood theory.

The formation of aggregates by the PMB monomer of phosphorylase *a* is hard to explain. By normal criteria the monomer is not to be regarded as a denatured protein. The absorption spectrum is identical with that of native phosphorylase *a*, the pyridoxal phosphate contribution being unchanged (*cf.* changes in its spectrum on denaturation, discussed by Krebs and Fischer, 1962).

The sedimentation coefficients for dimer and tetramer are as for the native species, as is the appearance in the electron microscope (the denatured state again appearing quite different). Moreover, as has been shown, the PMB-treated protein still interacts with AMP. It is therefore unlikely that unfolding effects have led to the appearance of new binding sites. Rather, it may be supposed that hitherto undetected binding sites are present in the native protein, but are associated with much weaker binding than the established sites.

We define the following binding sites: $\alpha\alpha$''s are the primary sites, which link the subunits in the dimer. These are strong in the native protein. The $\beta\beta'$ sites are responsible for the dimer $\rightleftharpoons$ tetramer equilibrium, are relatively weak, and are strengthened when AMP is bound to the protein. For isologous association, as in most globular proteins, species such as trimers and pentamers cannot be formed with these sites alone. It must therefore be supposed that further sites are present under our conditions. It seems improbable that these should be created by the attachment of the mercuribenzoate groups, and it seems more likely that they are in fact weak sites which become comparable with the $\alpha\alpha'$ and $\beta\beta'$ sites when these become drastically weakened by PMB. Since the polymerization occurs only at low ionic strengths, charged groups are evidently implicated.

With multiple binding sites and permitting a small degree of geometrical distortion, the possibilities exist of generating linear, branched, or cyclic structures. Caspar (1963) has discussed the preferential formation of closed structures by TMV protein and has rationalized this phenomenon in terms of the increased negative enthalpy of association on ring closure, which overcomes a small entropy loss. There is no reason why these arguments should not have a quite general applicability, and it may be supposed that a similar situation obtains in the present case. We cannot determine whether the $\gamma\gamma'$ sites are involved in the polymerization, or indeed whether there is a heterologous interaction, for example, of the modified α or β sites with each

other, or with different sites. In any event, the formation of closed shells is not surprising in this light.

Our hypothesis of additional sites carries with it the corollary that the native protein under the right conditions should aggregate to higher polymers by way of the various available sites. The end-to-end association of phosphorylase *a* dissolving from the crystalline state has already been noted (Figure 7). Further, high molecular weight aggregates of phosphorylase, not involving unfolding of the protein have been observed, for example, in phosphorylase *a* at high salt concentration (Wang and Graves, 1963), and in the absence of thiols (Seery *et al.*, 1967), and in the cold-aggregated form of phosphorylase *b* (Graves *et al.*, 1965). In both these last two cases a broad boundary in the ultracentrifuge, sedimenting at 20 S, has been reported. It may finally be noted that the multiple electrophoretic components found by Huang and Madsen (1966) to be formed by carbamylated phosphorylase present precisely the appearance of polymers. The electrophoretic migration distances of these zones fit satisfactorily onto a curve parallel to that shown in Figure 3. It thus appears that several sets of associating sites do in fact exist in the native phosphorylases, which make possible extensive changes in aggregation state in response to adjustments of the medium.

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