Structural Changes in Glycogen Phosphorylase As Revealed by Cross-Linking with Bifunctional Diimidates: Phospho–Dephospho Hybrid and Phosphorylase *a*†

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ABSTRACT: The technique of cross-linking with a series of bifunctional diimidates (maximal effective length ranging from 3.7 to 14.5 Å), followed by dodecyl sulfate gel electrophoresis, was applied to compare the subunit contact areas of phosphorylases *b*, *ab* (the phospho–dephospho hybrid), and *a* and to study the structure and ligand-induced structural changes in phosphorylases *ab* and *a*. Similarly to phosphorylase *b*, the nearest cross-linkable lysyl-NH_2 groups are about 3.7 Å apart across the intradimer subunit interface (contact *m*) and about 8 Å apart across the interdimer interface (contact *d*) in both phosphorylases *ab* and *a*. The activation of phosphorylase *b* induced by phosphorylation and that elicited by AMP binding are distinguishable at both contacts *m* and *d*. Phosphorylases *ab* and *a* tend to form tetramers whose structures are not identical, but AMP renders phosphorylase *ab* similar to phosphorylase *a*. Glucose, caffeine, and glycogen are able to dissociate both *a* and *ab* tetramers to dimers, whereas glucose 6-phosphate can only dissociate phosphorylase *ab*. The structure around the nucleotide site of phosphorylase *a* is rigid so that ligands binding here, such as AMP, ATP, ADP, inosine monophosphate, and glucose 6-phosphate, fail to influence the cross-link pattern. In contrast, in phosphorylase *ab* contact *m* is markedly affected by AMP, ATP, and glucose 1-phosphate; hence, in this respect phosphorylase *ab* resembles phosphorylase *b*.

In a previous work (Hajdu et al., 1979) we have applied the technique of cross-linking with bifunctional diimidates to study the structure and structural changes in phosphorylase *b*. By use of a series of diimidates of different lengths, the distances between lysyl-ε-NH_2 groups located near the intersubunit contact areas could be measured and ligand-induced conformation changes could be characterized in terms of differences in the cross-link patterns. In the present paper we describe results obtained by this simple technique with the phosphorylated forms of phosphorylase.

On the way of phosphorylating phosphorylase *b* by the specific kinase, as well as during the dephosphorylation of phosphorylase *a* by phosphatase, a phospho–dephospho hybrid (phosphorylase *ab*) is transiently formed (Hurd et al., 1966; Heilmeyer et al., 1970; Bot et al., 1974; Busby & Radda, 1976). This hybrid is enzymatically active and its activity is markedly influenced by ligands (Hurd et al., 1966; Gergely et al., 1974). It has been suggested that phosphorylase *ab* is a physiologically significant enzyme species (Gergely et al., 1974). The activity of the fully phosphorylated form, phosphorylase *a*, is only slightly affected by ligands (Graves & Wang, 1972), but ligand binding plays an important role in the control of both dephosphorylation (Nolan et al., 1964; De Wulf et al., 1970; Bot & Dôsa, 1971; Martensen et al., 1973a,b; Detwiler et al., 1977; Bot et al., 1978) and dimer–tetramer equilibrium (Graves & Wang, 1972).

As will be seen below, the characteristic alterations in the cross-link patterns induced by phosphorylation and by ligand binding to the phosphorylated enzyme forms may contribute to the elucidation of structural events taking place in the phosphorylase molecule.

Materials and Methods

Rabbit muscle phosphorylase *b* was prepared and recrystallized 3 times according to Fischer & Krebs (1962); it was converted to phosphorylase *a* as described by Krebs & Fischer (1962). Enzyme activity was assayed by the method of Illingworth & Cori (1953). Specific activity was 820 mkat kg^{-1} (i.e., 49 units mg^{-1}) for phosphorylase *b* and 890 mkat kg^{-1} (i.e., 53 units mg^{-1}) for phosphorylase *a*. Phosphorylase *ab*, a gift of Dr. Edit F. Kovács (Institute of Medical Chemistry, University of Medicine, Debrecen), was prepared and assayed according to Bot et al. (1974). Its specific activity was 835 mkat kg^{-1} in the presence of 1 mM AMP and 5 mM caffeine and 450 mkat kg^{-1} in the absence of effectors, which proved that it was a phospho–dephospho hybrid. All three types of phosphorylase preparations were treated with Norit A to remove AMP.

Cross-linking was performed, if not otherwise stated, with 0.1 mg/mL enzyme [0.5 μM in dimer, M_r 200 000 (Cohen et al., 1971)] in 0.2 M triethanolamine buffer, pH 8.0, at 30 °C, with 20 mM diimidates, except dodecanoic diimidate, which was 2 mM, for 60 min.

All other methods including protein determination, preparation of bifunctional diimidates, sodium dodecyl sulfate gel electrophoresis, and densitometry were the same as before (Hajdu et al., 1979). The two cross-link parameters, also described in detail by Hajdu et al. (1979), derived from the gel electrophoretic patterns were as follows:

$$r_k = \frac{k_L}{k_0}$$

where k_L and k_0 are the apparent first-order rate constants of the disappearance of the monomeric band as detected in the gel electrophoretic pattern in the presence and absence of a given ligand or with and without phosphorylation, respectively. The other parameter is

$$C_d = \frac{\text{tri} + \text{tetra}}{\text{total}} \times 100$$

where tri, tetra, and total mean the amount of protein found in the trimeric, tetrameric, and all bands, respectively.

Results and Discussion

Comparison of the Cross-Link Patterns of Active Phosphorylase Forms. The cross-link patterns of phosphorylases

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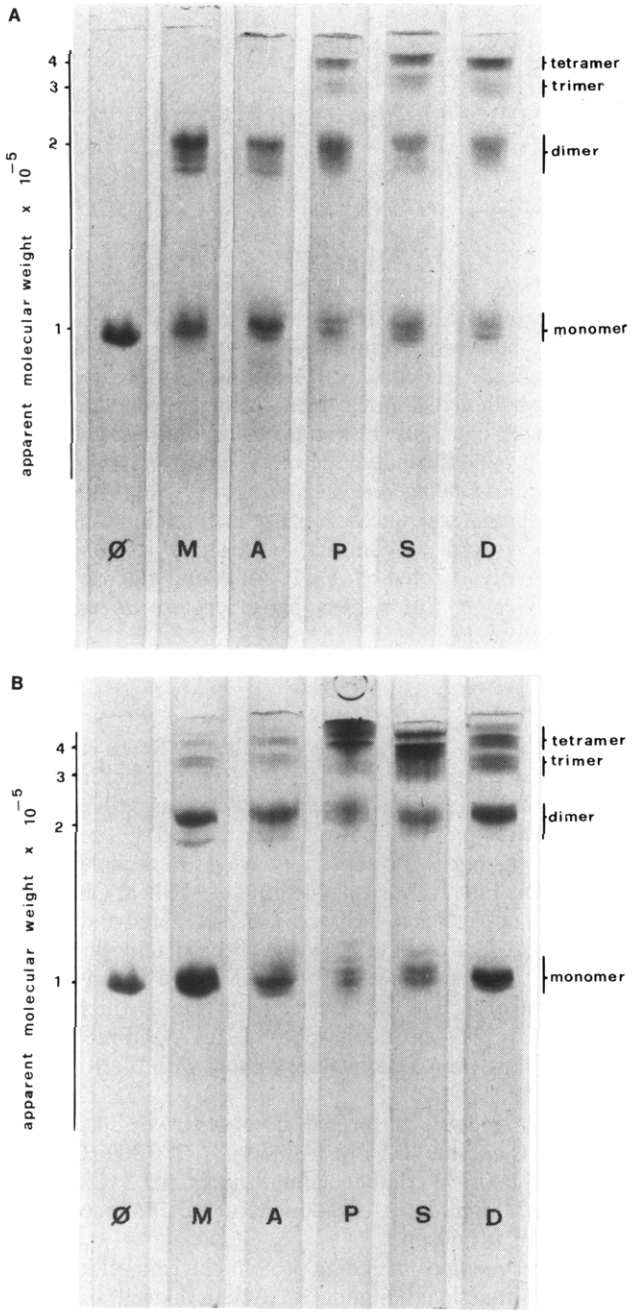

FIGURE 1: Dodecyl sulfate gel electrophoretic patterns of phosphorylase *ab* (A) and phosphorylase *a* (B) cross-linked with various diimidates. Cross-linking was carried out as described under Materials and Methods. *ϕ*, *un-cross-linked control*; M, A, P, S, and D, samples cross-linked with malonic, adipic, pimelic, suberic, and dodecanoic diimidate, respectively. The maximal effective reagent lengths of the various diimidates were as follows: malonic, 3.7 Å; adipic, 7.3 Å; pimelic, 8.5 Å; suberic, 9.7 Å; dodecanoic, 14.5 Å.

ab and *a* obtained with various diimidates are shown in Figure 1. It is seen that in the phosphorylated enzyme species already the shortest reagent, malonic diimidate (3.7 Å), is capable of producing cross-links across the intradimer subunit interface (contact *m*), just as it was for phosphorylase *b* (Hajdu et al., 1979). The interdimer interface (contact *d*) can be spanned only by reagents longer than ~8 Å, again similarly to phosphorylase *b*. On the other hand, unlike ligand-free phosphorylase *b*, the phosphorylated forms yield a great amount of tetramer, which is in accord with the enhanced propensity for tetramer formation of these species (Hurd et al., 1966). It should be mentioned that Hedrick et al. (1969) could not detect tetrameric phosphorylase *a* by gel electrophoresis in a nondissociating medium and Steiner et al. (1979) did not notice any tetramer in sedimentation runs of phosphorylase *b* plus 1 mM AMP. The discrepancy between these and our data may originate, apart from the nonidentical reaction conditions, from the possible dissociation of phosphorylase tetramer during electrophoresis or sedimentation [cf. Harrington & Kegeles (1973)], which in our experiments could not happen owing to covalent coupling. The cross-linking technique is a sensitive tool for the detection of tetrameric phosphorylases, as already observed by Pfeuffer et al. (1972). Nevertheless, one must bear in mind that covalent modification may shift association–dissociation equilibria in an unpredictable manner.

The monomer and dimer bands are split with several reagents as seen in Figure 1, which is due to the formation of cross-link isomers (Hajdu et al., 1977). The fine structure of dimer band has been analyzed in detail (Gusev et al., 1979) and was found to reflect the motility of the N-terminal tail. Namely, in phosphorylase *b* the N-terminal 18 residues are motile (Weber et al., 1978), whereas the same segment is immobilized in phosphorylase *a* (Fletterick et al., 1976; Madsen et al., 1978). The fact that more subbands can be seen in the monomer area of phosphorylase *a* (Figure 1) than could be discerned in the monomer band of phoshorylase *b* (Hajdu et al., 1979) suggests that the tail lysines (Lys-9 and Lys-11) are involved in intrachain cross-links in phosphorylase *a*.

The distance diagrams of cross-linking, i.e., the cross-link parameters C_d and r_k plotted vs. the maximal effective reagent lengths, for the various phosphorylase species are given in Figure 2. It is conspicuous from the C_d values that phosphorylase *a* has a marked tendency to form tetramers. The intermediate phosphorylase *ab* is clearly distinguishable from both phosphorylases *b* and *a*. The difference between the phosphorylated forms may arise from their dissimilar propensities for tetramer formation (Hurd et al., 1966). It is interesting to note that in the presence of 0.3 mM AMP the C_d diagram of phosphorylase *b* becomes similar to that of AMP-free phosphorylase *ab*, whereas the diagram of phosphorylase *ab* approaches that of phosphorylase *a*. The pattern of the fully phosphorylated form is hardly affected by AMP (cf. below). Since with phosphorylase *b* even as high as 1 mM AMP could not give the pattern characteristic of phosphorylase *a*, it seems that the tetramers produced by phosphorylation and by AMP are distinguishable. On the other hand, AMP can induce a phosphorylase *a* like tetramer structure in phosphorylase *ab*.

The effect of phosphorylation and AMP on overall cross-linkability is illustrated by the distance diagram of Figure 2B. Here the r_k parameters reflect changes relative to phosphorylase *b*. The differences between phosphorylase *b*–AMP, phosphorylase *ab*, and phosphorylase *a* are prominent mainly at the short reagents, which report on the intradimer interface, contact *m*. (The differences seen at contact *d* in Figure 2A are probably obscured here by the complexity of r_k.) It follows that the conformational changes brought about by phosphorylation and AMP are also distinguishable at contact *m*. This is in accord with the finding that the phosphorylase *b*–AMP complex displays homotropic cooperativity while phosphorylase *a* lacks this property (Graves & Wang, 1972).

We may conclude that incorporation of one phosphoryl group into phosphorylase *b* already "opens" the *d* interface giving rise to tetramers. AMP tightens this tetramer, rendering it similar to phosphorylase *a*. The structure at contact *m* is markedly different in phosphorylases *a* and *b*.

Table I: Effect of Ligands on the Cross-Link Parameter C_d of Phosphorylase *ab*[a]

diimidate	maximal effective reagent length (Å)	C_d without ligand	0.3 mM AMP	5 mM IMP	5 mM ADP	5 mM ATP	10 mM Glc-1-P	50 mM Glc-6-P	50 mM glucose	5 mM caffeine	1% glycogen
pimelic	8.5	24 ± 5	30 ± 3	20 ± 4	25 ± 7	18 ± 1	24 ± 4	10 ± 3	2 ± 1	1 ± 1	9 ± 3
suberic	9.7	31 ± 2	48 ± 8	34 ± 6	41 ± 9	31 ± 6	33 ± 1	26 ± 4	8 ± 5	4 ± 1	23 ± 14
dodecanoic	14.5	46 ± 1	44 ± 5	40 ± 5	38 ± 4	38 ± 1	43 ± 8	28 ± 5	16 ± 7	12 ± 3	11 ± 7

[a] Cross-linking was performed at 30 °C as described under Materials and Methods at the ligand concentrations indicated. The mean ± standard deviation of three independent experiments is shown.

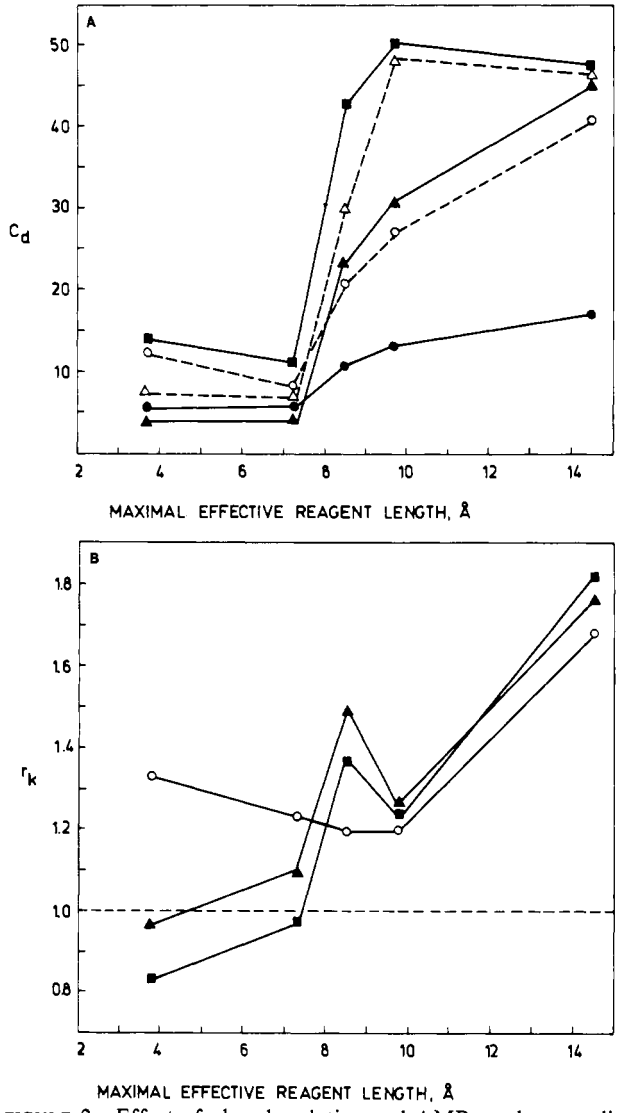

FIGURE 2: Effect of phosphorylation and AMP on the cross-link parameters of phosphorylase. (A) C_d distance diagram. Symbols: (●) phosphorylase *b*; (○) phosphorylase *b* plus 0.3 mM AMP; (▲) phosphorylase *ab*; (△) phosphorylase *ab* plus 0.3 mM AMP; (■) phosphorylase *a*. The points are the mean of three independent experiments. The error, not shown for the sake of clarity, was similar to that in Tables I and II. Note that the abscissa is discontinuous; the lines connecting the points only serve better visualization. (B) r_k distance diagram. Reagent lengths and abscissa are as in (A). The r_k values were calculated by taking the rate constants measured with phosphorylase *b* as k_0. Symbols are as in (A). The points are the mean of three independent experiments; the error was similar to that in Figure 3.

Effect of Ligands on the Cross-Link Pattern of Phosphorylase ab. The effect of ligands on the dimer ⇌ tetramer transition of phosphorylase *ab* is shown in Table I. Only reagents that gave C_d values higher than the "noise" level (up to about $C_d = 8$) are included. As already demonstrated above

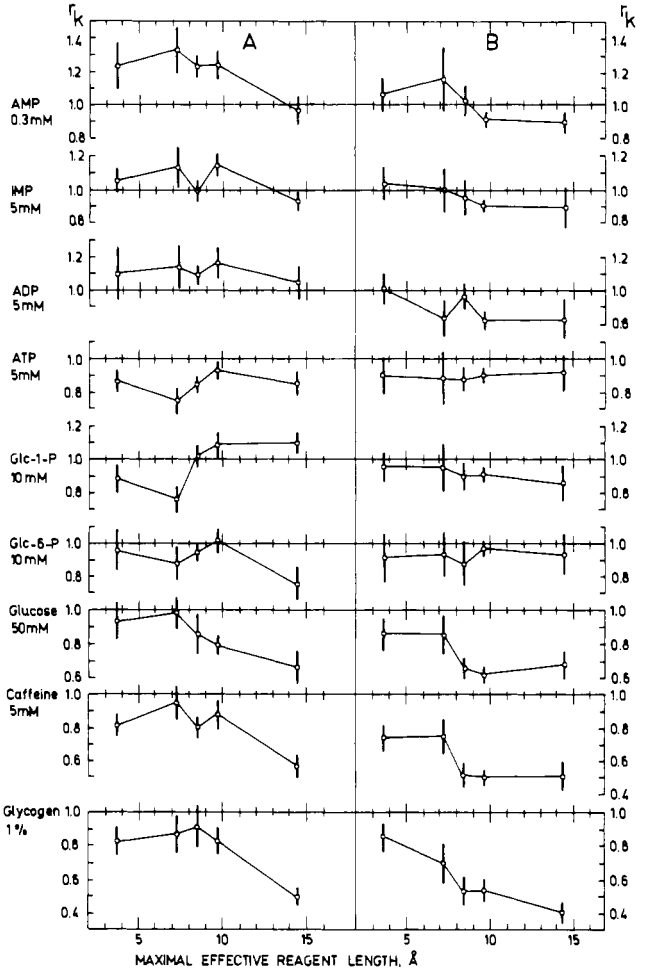

FIGURE 3: Effect of ligands on the r_k cross-link parameter of phosphorylase *ab* (A) and phosphorylase *a* (B). Cross-linking was carried out at 30 °C in (A) and at 18 °C in (B), at the ligand concentrations indicated. The abscissa is discontinuous (cf. Figure 2). The mean ± standard deviation of three independent experiments is shown.

(Figure 2A), AMP increases cross-linking with pimelic and suberic diimidates. The other nucleotides have no effect. Likewise, 10 mM Glc-1-P[1] did not change the C_d values. It should be noted that Hurd et al. (1966) observed an increase in the amount of tetramer at 75 mM Glc-1-P. We did not use that high Glc-1-P concentration to avoid the reaction between reagent and ligand from becoming significant. Glc-6-P shifts the equilibrium toward dimers (Hurd et al., 1966) but not as pronouncedly as do glucose, glycogen, and caffeine. The dimer-forming effect of the last three ligands has already been observed with phosphorylase *a* (Wang et al., 1965a,b; Bot et al., 1977) and also with phosphorylase *b* (Hajdu et al., 1979).

[1] Abbreviations used: IMP, inosine 5′-monophosphate; Glc-1-P, glucose 1-phosphate; Glc-6-P, glucose 6-phosphate.

Table II: Effect of Ligands on the Cross-Link Parameter C_d of Phosphorylase a[a]

diimidate	maximal effective reagent length (Å)	C_d without ligand	0.3 mM AMP	5 mM IMP	5 mM ADP	5 mM ATP	10 mM Glc-1-P	10 mM Glc-6-P	50 mM glucose	5 mM caffeine	1% glycogen
pimelic	8.5	57 ± 2	54 ± 9	57 ± 5	47 ± 4	49 ± 3	53 ± 6	53 ± 1	28 ± 10	10 ± 9	10 ± 8
suberic	9.7	60 ± 3	51 ± 4	52 ± 4	47 ± 1	54 ± 2	58 ± 5	60 ± 3	27 ± 3	10 ± 1	26 ± 18
dodecanoic	14.5	41 ± 8	38 ± 5	35 ± 7	30 ± 1	33 ± 1	31 ± 3	41 ± 1	22 ± 7	14 ± 5	6 ± 3

[a] Cross-linking was performed at 18 °C as described under Materials and Methods at the ligand concentrations indicated. The mean ± standard deviation of three independent experiments is shown.

Figure 3A illustrates the effect of ligands on overall cross-linking in terms of r_k values, which will be discussed in comparison to the picture obtained with phosphorylase *b* (Hajdu et al., 1979). AMP furthers cross-linking primarily with the shorter reagents, as observed with phosphorylase *b*. Its diminishing effect toward the longer reagents is probably due to the circumstance that unliganded phosphorylase *ab* is already largely in the tetramer state. IMP has scarcely any influence on cross-linking. Apparently, the IMP-induced active conformation is distinguishable from that established by AMP not only in phosphorylase *b* (Morange et al., 1976) but also in the hybrid. The cross-link patterns obtained with ATP and Glc-1-P are essentially the same as in the case of phosphorylase *b*, characterized by a minimum at adipic diimidate. ADP, though it only slightly affects cross-linking, gives a pattern distinct from that found with phosphorylase *b* or phosphorylase *a* (cf. below). The reason of this nonintermediate behavior is not known; it is presumably due to some special feature of phosphorylase *ab*. It should be recalled that ADP tends to decompose, giving rise to AMP, which may be related to the anomalous pattern. However, even if so, the nonintermediate behavior of the hybrid is still to be invoked, albeit not strictly toward ADP but rather toward a mixture of ADP, AMP, and inorganic phosphate. Glc-6-P was previously shown not to influence the r_k values of phosphorylase *b*; the same holds for the hybrid with the exception of the longest reagent, which reflects the tetramer-dissociating effect already seen in Table I. In accord with the same dissociating influence of glucose, caffeine, and glycogen, these ligands decrease the r_k values with the longer reagents. Here the only significant difference from phosphorylase *b* is that caffeine decreases, rather than increases, the r_k with malonic and adipic diimidates.

The above data indicate that in phosphorylase *ab* the conformation near contact *m* can be influenced by AMP, ATP, and Glc-1-P, which is consistent with the fact that the activity of the enzyme is also affected by these ligands (Hurd et al., 1966; Gergely et al., 1974). The similarity of contact *m* in phosphorylases *ab* and *b* is further indicated by the r_k values of phosphorylase *ab* with the short reagents, which are close to unity (Figure 2B).

Effect of Ligands on the Cross-Link Pattern of Phosphorylase a. Phosphorylase *a* was cross-linked in the presence of ligands at 18 °C in order to characterize the dimer ⇌ tetramer transition under conditions used in earlier investigations. Table II shows the C_d values with the long reagents that can span contact *d*. The data are in full agreement with earlier results obtained by other methods. AMP no longer promotes tetramer formation; its slight dissociating effect (Wang & Graves, 1964) is hardly noticeable because of the experimental error. Likewise, Glc-1-P and Glc-6-P have no significant effect either (Hurd et al., 1966; Helmreich et al., 1967). Glucose, glycogen, and caffeine shift the dimer ⇌ tetramer equilibrium toward the dimer (Wang et al., 1965a,b; Bot et al., 1977).

The effect of ligands on overall cross-linking is illustrated in the r_k diagrams of Figure 3B. Ligands binding to the nucleotide site, such as AMP, IMP, ADP, ATP, Glc-1-P, and Glc-6-P (Johnson et al., 1978; Kasvinsky et al., 1978), exert hardly any influence on the cross-linking of phosphorylase *a*. The lack of responsiveness is not due to the lower temperature (18 vs. 30 °C), since phosphorylase *b* exhibits cooperative conformational changes even at 4 °C (Morange et al., 1976). This corroborates the fact that the incorporation of two phosphoryl groups makes the subunit contact area rigid, and the allosteric nucleotide site becomes "desensitized" (Graves & Wang, 1972). The decrease of r_k at the longer reagents in the presence of dimer-forming ligands (glucose, glycogen, and caffeine) is due to the dissociation of the tetramer. This is in harmony with the finding that the very same effectors facilitate the reaction of phosphorylase phosphatase at low temperature since phosphatase is unable to attack phosphorylase *a* (Bot & Dősa, 1971; Martensen et al., 1973b; Bot et al., 1977). Glc-6-P does not seem to dissociate the enzyme, and, indeed, it does not enhance the phosphatase reaction at 18 °C (E. Kovács, personal communication).

According to recent X-ray diffraction studies, phosphorylase *a* crystals grown in the presence of glucose shatter when soaked in a solution of thioglucose 1-phosphate owing to the structural rearrangement that occurs on binding the substrate analogue (Madsen et al., 1978). The differences observed in the r_k and, more pronouncedly, in the C_d values with glucose and Glc-1-P are in agreement with this finding.

Since the symmetry of a tetrameric protein can be deduced from cross-linking data if other aggregational forms are practically negligible, we cross-linked phosphorylase *a* in 1 mg/mL concentration at 18 °C with various amounts of diimidates. The ratios of rate constants of cross-linking across the two types of intersubunit contacts, i.e., the so-called k_p/k_q values (Hajdu et al., 1976), were as follows: 4.2 ± 0.5, 3.0 ± 0.4, 2.3 ± 0.7, 1.8 ± 0.4, and 1.4 ± 0.2 for malonic, adipic, pimelic, suberic, and dodecanoic diimidates, respectively. As all ratios significantly differ from unity, phosphorylase *a* proves to be an isologous (D_2) tetramer. This is in accord with available X-ray evidence (Fletterick et al., 1976; Bartels & Colman, 1976).

In conclusion, some of the major structural transitions that could be monitored by the cross-link technique in rabbit muscle phosphorylase are schematically shown in Figure 4. Predominantly dimeric phosphorylase *b* can be converted to tetramers either by liganding with AMP or by partial phosphorylation. The two types of tetramer have distinguishable conformations. The combination of AMP and partial phosphorylation produces a phosphorylase *a* like tetramer. The fully phosphorylated form, with or without AMP, is a fairly rigid structure in which subunit interactions do no propagate through contact *m*. This scheme is undoubtedly an oversimplification and reflects detectability in vitro rather than reality in vivo. Main structural forms exercise their effects on these

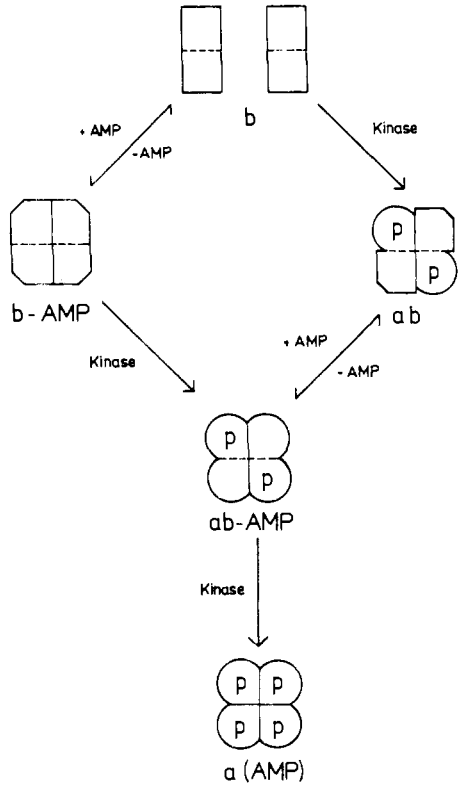

FIGURE 4: Structural states detectable by cross-linking in rabbit muscle phosphorylase. Letters a, b, and ab denote the respective forms of phosphorylase. Square, truncated square, and circular protomer symbols designate different polypeptide conformations. Protomer interfaces indicated by the dotted line denote allosterically competent subunit contact *m*. "Kinase" means the incorporation of phosphate (P) at Ser-14 by phosphorylase kinase.

other ligands which eventually tailor the enzyme to respond to the metabolic need in the muscle cell.

Acknowledgments

The authors express their gratitude to Dr. Edit F. Kovács (Institute of Medical Chemistry, University of Medicine, Debrecen) for kindly supplying phosphorylase *ab* and for letting us know unpublished results. Thanks are due to Ágnes Németh and András Ujházy for the skillful technical assistance.

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