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Structural Changes in Glycogen Phosphorylase As Revealed by Cross-Linking with Bifunctional Diimidates: Phosphorylase *b*†

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ABSTRACT: Glycogen phosphorylase *b* was cross-linked with a homologous series of diimidates (maximal effective lengths, 3.7–14.5 Å) in the presence of various activators, inhibitors, and substrates under conditions where no polymers were formed. The cross-link products were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the gels were evaluated by densitometry. From the changes of cross-link patterns the following main conclusions were drawn. (1) In dimeric phosphorylase *b* there are at least two lysyl pairs at the subunit interface (contact m) whose ϵ-NH_2 groups are within 3.7 Å. (2) When dimeric phosphorylase *b* associates to form tetramers, the nearest two lysyl ϵ-NH_2 groups are ~8 Å apart at the interdimer interface (contact d). (3) The conformational change caused by adenosine 5′-monophosphate (AMP) promotes cross-linking at contact m and induces tetramerization of the enzyme with consequential cross-linkability at contact d. (4) In contrast to AMP, the other activator, inosine monophosphate, does not elicit any change in cross-linking. (5) Adenosine 5′-diphosphate (ADP) and adenosine 5′-triphosphate (ATP) diminish cross-link formation between NH_2 groups located within a distance of 4–9 Å at contact m. (6) Glucose 1-phosphate mimicks the effect of ADP, but when applied together with AMP it amplifies the effect of the latter (heterotropic interaction). (7) Glucose, caffeine, and glycogen decrease cross-link formation with the longer reagents owing to the dissociation of tetrameric phosphorylase *b*. The binding of glucose 6-phosphate does not seem to induce structural changes detectable in the cross-linkability of contact m. All four effectors diminish the influence of AMP.

It has been a long-lasting challenge to elucidate the structural basis of the catalytic and regulatory properties of glycogen phosphorylase (EC 2.4.1.1), the key enzyme of glycogen breakdown. Important advances have recently been made to this end: the structure of both phosphorylase *a* (Fletterick et al., 1976) and phosphorylase *b* (Weber et al., 1978) has been solved at 3-Å resolution, and the complete amino acid sequence of the phosphorylase subunit (841 residues) has been determined (Titani et al., 1977). The binding sites for the various substrates and effectors have been located in the three-dimensional structure, and the details of the transition from the inactive to the active conformation (Helmreich et al., 1967; Buc, 1967) are beginning to take shape (Weber et al., 1978; Kasvinsky et al., 1978a,b; Dreyfus et al., 1978).

Cross-linking with bifunctional reagents followed by sodium dodecyl sulfate gel electrophoresis, a fairly simple chemical technique, has proved to be a useful tool in the study of oligomeric enzymes as regards the number and arrangement of subunits (Davies & Stark, 1970; Hajdu et al., 1976). We deemed that this method might provide information, complementary to that available, about the structural events in phosphorylases on the following grounds.

(1) By using a homologous series of diimidates, we can measure interlysyl distances on the surface of a protein. Namely, the cross-linking of a given lysine pair by such a reagent will be influenced, among others, by the distance and reactivity of the ϵ-NH_2 groups. It is expected that a conformational change involving these lysines will affect the propensity for cross-linking in a different manner for reagents of various lengths by altering NH_2 group distances and/or reactivities.

(2) Allosteric transitions propagating from one subunit to the other are bound to include structural changes near the subunit contacts, and cross-linking reports mainly about this area. The allosteric nucleotide binding site in phosphorylase is also near the subunit contact region (Fletterick et al., 1976; Weber et al., 1978).

(3) Cross-linking data, in contrast to the X-ray picture, are related to the protein in solution; hence, they may reflect the dynamics of protein structure. Clearly, cross-linkability of a lysine pair will also be influenced by the movements of the two side chains relative to each other.

(4) In knowledge of the three-dimensional structure of phosphorylase, tentative assignments can be made to some cross-links which may later be checked by X-ray analysis. In

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this way the molecular movements resulting in a change of cross-linkability might be pinpointed.

In this paper we describe the cross-linking of phosphorylase *b*. This enzyme is known to be active only in the presence of AMP or IMP;[1] its activity is allosterically inhibited by ATP, ADP, Glc-6-P, caffeine, and glucose [cf. Graves & Wang (1972)]. We have found that these effectors, as well as two substrates, Glc-1-P and glycogen, characteristically influence cross-linking, from which inferences can be made as to the structure and conformational changes of the enzyme.

Materials and Methods

Rabbit skeletal muscle phosphorylase *b* was prepared according to Fischer & Krebs (1962), recrystallized three times, and freed from nucleotide by Norit A treatment (Fischer & Krebs, 1958). The A_{260}/A_{280} ratios were in the range of 0.53–0.56 after the charcoal treatment. The specific activity of the enzyme was 820 mkat kg^{-1} (i.e., 49 units mg^{-1}) as determined by the method of Illingworth & Cori (1953) with 16 mM Glc-1-P and 1 mM AMP. Protein content was measured spectrophotometrically at 280 nm by using the absorption coefficient 1.30 cm^2 mg^{-1} (Kastenschmidt et al., 1968). Glc-1-P and glycogen were prepared according to McCready & Hassid (1955) and Helmreich & Cori (1964), respectively; both were treated with Norit A. The bifunctional diimidoester hydrochlorides were prepared by the procedure of McElvain & Schroeder (1949) from the corresponding dinitriles. All other chemicals were commercial preparations of reagent grade.

Cross-Linking of Phosphorylase b. As a rule, 0.18 mg/mL enzyme [0.9 μM in dimer, M_r 200 000 (Cohen et al., 1971)] was preincubated with or without ligands for 10 min at 30 °C in 0.2 M triethanolamine buffer, pH 8.0. The reaction was started by the addition of diimidoesters in a final concentration of 20 mM, except dodecanedioic diimidate, which was 2 mM. Cross-linking was allowed to proceed for 60 min at 30 °C, and then the reaction was stopped by lowering the pH to 7.0.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Fairbanks et al. (1971), staining with Coomassie brilliant blue R-250, and the densitometric evaluation of gels were performed as described earlier (Hajdu et al., 1976). Cross-linking did not change the staining properties of the protein.

Treatment of Densitometric Data. To characterize cross-linking quantitatively, two parameters were calculated from the densitometric data. The *rate constant ratio of cross-linking*, r_k, is defined as $r_k = k_L/k_0$, 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 [cf. Hajdu et al. (1976)] in the presence and absence of a given ligand, respectively. Since all samples were cross-linked for the same time under identical conditions, the value of r_k could be calculated as $r_k = \ln (\text{monomer/total})_L / \ln (\text{monomer/total})_0$ by measuring the amount of protein in the monomer band on gel electrophoresis and the sum of protein in the monomer, dimer, trimer, and tetramer bands, i.e., the total amount of protein in the gel. The latter was usually ≥90% of the amount of protein loaded onto the gel. Subscripts L and 0 refer to data obtained in the presence and absence of a given ligand, respectively. The value of r_k can be regarded as a measure of the effect of a ligand on the overall propensity for cross-linking of the enzyme with a given bifunctional reagent. Thus, if $r_k > 1$ the ligand promotes cross-linking, whereas if $r_k < 1$ the ligand hinders cross-link formation.

The other cross-link parameter, C_d, is defined as C_d = (trimer + tetramer)/total × 100, i.e., the percentage amount of protein found in the trimer and tetramer bands. It should be mentioned that C_d values obtained with different diimidates cannot be strictly compared because of the somewhat unlike hydrolysis rates of reagents.

Since phosphorylase *b* may form tetramers, two types of intersubunit cross-link should be distinguished: one *within* dimers (across the monomer–monomer interface, referred to as contact m) and one *between* dimers (across the dimer–dimer interface, contact d). Hence, C_d is a measure of the cross-linkability of contact d, whereas r_k refers to the cross-linking of both contacts m and d.

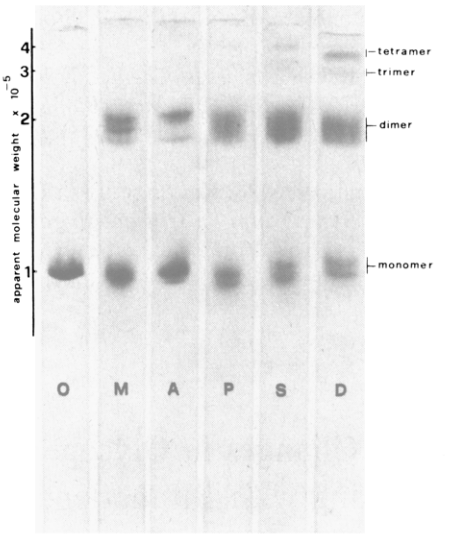

FIGURE 1: Sodium dodecyl sulfate gel electrophoretic patterns of phosphorylase *b* cross-linked with various diimidates. For experimental conditions, cf. Materials and Methods. O stands for un-cross-linked phosphorylase *b*, whereas M, A, P, S, and D stand for enzyme cross-linked with malonic, adipic, pimelic, suberic, and dodecanedioic dimethylimidate, respectively. The maximal effective reagent lengths for malonic, adipic, pimelic, suberic, and dodecanedioic diimidates are 3.7, 7.3, 8.5, 9.7, and 14.5 Å, respectively.

Results and Discussion

Cross-Linking of Phosphorylase b. Figure 1 shows the sodium dodecyl sulfate gel electrophoretic patterns of phosphorylase *b* samples cross-linked with various diimidates. It is seen that already the shortest reagent, malonic diimidate, produces covalent dimers, which indicates that there are lysyl residues within 3.7 Å distance from each other on the two sides of contact m. The dimer band produced by malonic diimidate is split, which is due to the formation of cross-link isomers (Hajdu et al., 1977). Although not very clearly, three dimeric subbands can be distinguished: two stronger ones with lower mobility and a weaker one with greater mobility. This suggests that more than one lysyl pair is involved in the intersubunit cross-linking of phosphorylase *b*. From the current knowledge of the three-dimensional structure of phosphorylase *b* (L. N. Johnson, personal communication), it seems probable that one of the major sites of these cross-links is between Lys-191 from the N-terminal domain of one subunit and Lys-41 from the "cap" region of the other subunit. The N-terminal tail of the polypeptide chain (about 20 residues), which is motile in phosphorylase *b* (Weber et al., 1978) and extends into the other subunit in phosphorylase *a* (Fletterick et al., 1976), also contains two lysines (9 and 11) that might be involved in cross-linking contact m. A detailed analysis of this possibility

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

Table I: Effect of Ligands on the C_d Value of Phosphorylase *b* Cross-Linking[a]

reagent	no addition	IMP (5 mM)	ADP (5 mM)	ATP (5 mM)	Glc-1-P (10 mM)	Glc-6-P (10 mM)	glucose (50 mM)	caffeine (5 mM)	glycogen (1%)
suberic diimidate	12 ± 4	11 ± 4	15 ± 6	6 ± 2	18 ± 1	12 ± 1	8 ± 2	6 ± 4	10 ± 2
dodecanedioic diimidate	15 ± 4	19 ± 6	20 ± 8	14 ± 6	15 ± 8	4 ± 3	5 ± 3	5 ± 2	5 ± 2
pimelic diimidate[b]	20 ± 7	7 ± 2	6 ± 2	8 ± 3	16 ± 6	3 ± 2	6 ± 3	4 ± 2	10 ± 4
suberic diimidate[b]	27 ± 8	16 ± 7	19 ± 4	16 ± 4	35 ± 2	10 ± 5	6 ± 3	4 ± 1	14 ± 2
dodecanedioic diimidate[b]	40 ± 9	33 ± 13	24 ± 8	35 ± 2	47 ± 5	14 ± 3	18 ± 1	11 ± 6	14 ± 2

[a] The mean ± standard deviation values from four to eight independent experiments are shown. [b] With 0.3 mM AMP.

has been described separately (N. B. Gusev, J. Hajdu, and P. Friedrich, unpublished experiments).

With adipic diimidate the intermediate dimer subband is missing, but a minor subband appears in the monomer area; the latter becomes more prominent with pimelic and suberic diimidates. It has already been discussed (Hajdu et al., 1977), as well as shown experimentally (Wermuth et al., 1979), that cross-link isomers may also ensue from intrapolypeptide cross-linking. It seems then that two lysyl residues, the cross-linking of which results in band splitting, are ~8 Å from each other in the phosphorylase monomer. With the longest reagents a small amount of trimer and tetramer bands also appears; thus, under the experimental conditions applied, a minor fraction of the enzyme is in the tetrameric state.

Effect of AMP on the Cross-Linking of Phosphorylase b. Since AMP is the main activator of phosphorylase *b*, which may also have physiological significance, the effect of this ligand was studied in greater detail. Figure 2 shows how different AMP concentrations influence the cross-link parameters C_d and r_k. In these *distance diagrams* the values of cross-link parameters are illustrated as a function of the maximal effective length of the reagent. Figure 2A clearly shows that trimers and tetramers are formed in appreciable amounts only if reagent length exceeds ~8 Å. (The C_d values of 5–10 measured with malonic and adipic diimidates should be regarded as the "noise" of the system which may originate from intermolecular cross-linking due to protein aggregation, etc.) Increasing concentrations of AMP increase the C_d values, but the saturation-type curves obtained when plotting C_d vs. AMP concentration (not shown) do not conform to a single binding constant. The inference from Figure 2A is that AMP brings about the tetramerization of phosphorylase *b* and the nearest two external (cross-linkable) lysyl groups across contact d are ~8 Å from each other. Since contact d is not known from X-ray work, the identification of these lysines would help in pinpointing the interdimer bonding domain.

The value of r_k is also raised by increasing AMP concentrations (Figure 2B), but here the concentration dependence is rather irregular. At low AMP concentration (0.1 mM) cross-linking is enhanced only with the shorter reagents, whereas at higher ligand concentrations the reaction of longer reagents, too, is markedly promoted.

AMP, a class I activator of phosphorylase *b* according to the classification of Morange et al. (1976), can bind to two sites on the phosphorylase monomer (Wang et al., 1965a; Kasvinsky et al., 1978a). The tight activator (nucleotide) site is close to contact m and to the Lys-41–Lys-191 pair. The r_k enhancement observed with short reagents is very probably due to a conformational change in this area which might be related to the tightening of monomer–monomer contact and the homotropic cooperativity of AMP (Remy & Buc, 1970; Buc-Caron & Buc, 1975). The increased r_k values obtained with long reagents presumably reflect tetramer formation induced by the saturation of nucleotide site, as already seen from the C_d values.

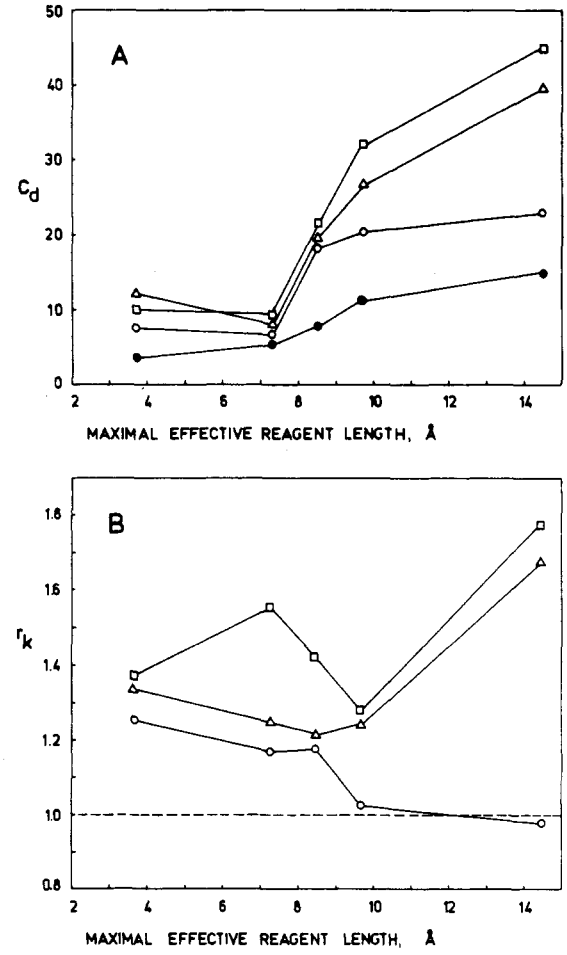

FIGURE 2: Effect of AMP on the cross-link parameters of phosphorylase *b*. (A) C_d as a function of maximal effective reagent length at various AMP concentrations: (●) without AMP; (○) 0.1 mM AMP; (△) 0.3 mM AMP; (□) 1.0 mM AMP. The points without AMP are the mean of seven independent experiments. The other points are the mean of at least four independent experiments. The error, not shown for the sake of clarity, was similar to that in Table I. The lines connecting the points have no physical meaning; they only serve for better visualization. (B) r_k as a function of reagent length at various AMP concentrations. The symbols and number of experiments are the same as in part A. The error, not shown for the sake of clarity, was similar to that in Figure 3.

Effect of Other Ligands and AMP–Ligand Combinations on the Cross-Linking of Phosphorylase b. The effects of a variety of ligands, with and without AMP, on the cross-link parameters are shown in Table I and Figure 3. The combination of other ligands with AMP was of interest because the active species is the phosphorylase *b*–AMP complex. In Table I only those cross-linkers were included with which the C_d values were above the "noise" level (cf. above). From the data the following inferences can be made.

IMP, a class II activator (Morange et al., 1976), does not alter the cross-link pattern of unliganded phosphorylase *b*, in

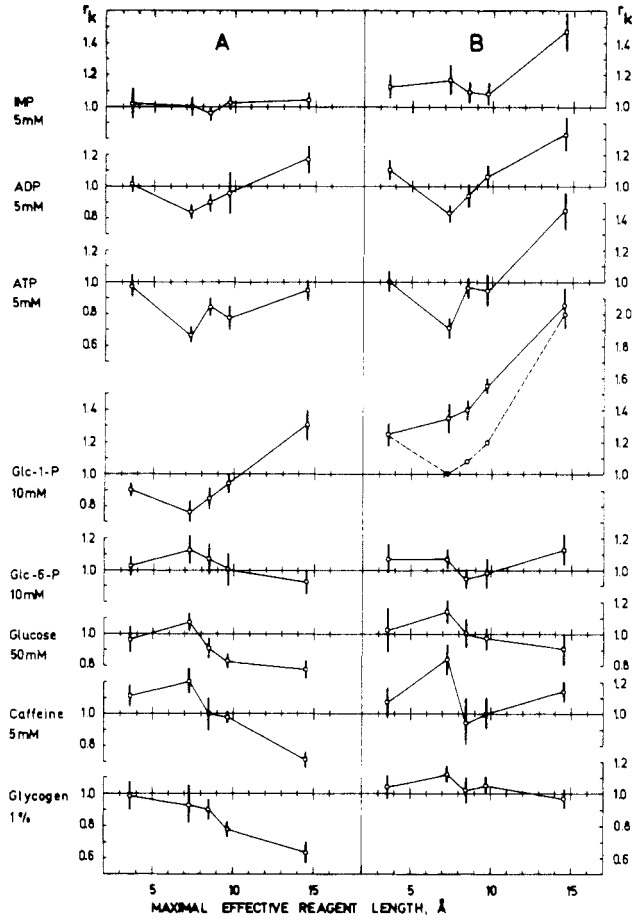

FIGURE 3: Effect of ligands on the r_k values of phosphorylase *b* cross-linking. The r_k values are shown as a function of maximal effective reagent length. (A) Cross-linking in the presence of a single ligand, as indicated; (B) cross-linking in the presence of 0.3 mM AMP + the ligand indicated. The points connected by dotted lines for Glc-1-P + AMP denote the calculated average of the r_k values obtained with the two ligands when applied separately. The mean ± standard deviation of four independent experiments is illustrated. The lines connecting the points only serve for better visualization (cf. Figure 2).

contrast to AMP. When both AMP and IMP were present, the latter decreased the effect of the former presumably by partially replacing it at the nucleotide site. These data are in qualitative agreement with earlier observations according to which the structural changes induced by AMP and IMP are quite different (Black & Wang, 1968; Ho & Wang, 1973). Indeed, IMP caused hardly any change in the parameters of various probes attached to the enzyme (Busby & Radda, 1976). On the other hand, the tetramer-dissociating effect expected from a class II activator (Morange et al., 1976) was not seen under the present conditions.

ADP and ATP are physiologically important effectors whose concentration in muscle ensures that, at the given AMP concentration, phosphorylase *b* should not be active. These two ligands have rather similar patterns characterized by decreased cross-linking, relative to the control, with medium length reagents, which are very much different from the AMP pattern. It appears that ADP and ATP induce a "wrong" conformation, thereby inhibiting the residual activity of phosphorylase *b* measured in the absence of AMP (Morange et al., 1976). When applied together with AMP, ADP and ATP seem to expel AMP from the nucleotide site, as shown by equilibrium dialysis (Avramovic & Madsen, 1968; Morange et al., 1976).

The substrate Glc-1-P, by itself, causes an alteration in cross-linking similar to that of ADP. This is probably due to its binding to the nucleotide site (Sygusch et al., 1977; Weber et al., 1978; Vandenbunder et al., 1978). On the other hand, if combined with AMP, Glc-1-P induces a very different transition (Figure 3B) which can be the manifestation of the heterotropic interaction between these ligands (Graves & Wang, 1972; Griffiths et al., 1976b). In this case, Glc-1-P presumably binds to the catalytic site for which it has a higher affinity (Weber et al., 1978). Our results are in good agreement with earlier data (Busby & Radda, 1976) according to which Glc-1-P and AMP, when applied separately, induce opposite conformational changes in phosphorylase *b* different from that when both are present.

Glc-6-P, a physiologically important inhibitor of the enzyme, did not significantly alter the cross-link pattern of the unliganded enzyme, but counteracted the effects of AMP. This inhibitor is held to bind close to the activator site (Johnson et al., 1978) but in a manner suggesting structure-mediated rather than direct competition with AMP (Buc-Caron & Buc, 1975). The tetramer-forming influence of AMP was also markedly reduced by Glc-6-P, which is to be expected if Glc-6-P only binds to the activator site. It should be mentioned that these cross-link data are apparently at variance with electron spin resonance (Griffiths et al., 1976a) and X-ray (Johnson et al., 1978) studies according to which Glc-6-P elicited the largest change ever experienced in the enzyme structure. It seems that these conformational changes do not involve the intersubunit contact area.

Glucose, as a competitive inhibitor, is bound at the catalytic site, whereas caffeine (and its analogues) is bound at the nucleoside site, which are quite far (30 and 40 Å, respectively) from the activator site (Kasvinsky et al., 1978b). These two inhibitors produce rather similar cross-link patterns; they seem to dissociate tetrameric phosphorylase *b*, even in the presence of AMP. The tetramer-dissociating effect of glucose (Wang et al., 1965a) and of caffeine (Bot et al., 1977) has only been described for phosphorylase *a*.

Glycogen, the physiological "support medium" of phosphorylase (Meyer et al., 1970), attaches to the enzyme at the so-called glycogen storage site, some 30 Å from the catalytic site and 40 Å from the nucleotide site, and is believed not only to anchor but also to activate the enzyme (Fletterick et al., 1976; Kasvinsky et al., 1978a; Weber et al., 1978). According to its cross-link pattern, glycogen dissociates unliganded tetrameric phosphorylase *b* and largely prevents tetramerization induced by AMP. In other respects, too, glycogen lessens the AMP effect. The tetramer-dissociating influence of glycogen has only been deduced from activity measurements (Wang et al., 1965b), and the moderation of AMP-induced conformational changes has been shown calorimetrically (Merino et al., 1977).

Throughout this paper changes in cross-linking on the effect of various ligands have been taken to reflect structural alterations of the enzyme. Although this working hypothesis cannot be rigorously proven, the following arguments support the validity of this interpretation. (1) The ligands do not react directly with the bifunctional diimidates. At any rate, such consumption of the reagents could only result in decreased cross-linking with all reagents. (2) Although it cannot be excluded that some ligands exert their effect, at least in part, by masking certain lysyl groups, in the majority of cases such direct hindrance seems improbable. AMP, IMP, and ATP bind to the same site (the activator site; Weber et al., 1978); still they produce markedly different cross-link patterns. Moreover, as in point 1, steric hindrance would not explain

cross-link enhancements. (3) Preliminary X-ray diffraction investigations indicate that cross-linking of phosphorylase *b* crystals with malonic diimidate does not cause major changes in the three-dimensional structure (L. N. Johnson, personal communication).

In conclusion, the study of cross-link parameters C_d and r_k, the distance diagrams of Figures 2 and 3, reveals a general feature, in addition to many specific ones, of the ligand-induced conformational changes in phosphorylase *b*. This is the multiplicity of conformations, as reflected in the distance and reactivity of lysyl ϵ-NH_2 groups. The activators AMP and IMP are characterized by very different cross-link patterns, the inhibitor Glc-6-P has about the same pattern as IMP, the substrate Glc-1-P mimicks the effect of ADP, an inhibitor, and AMP + Glc-1-P gives a cooperative change, not to mention glycogen, whose effect must be superimposed on all others in the glycogen particle (Meyer et al., 1970). Thus, a two-state (Monod et al., 1965; Buc-Caron & Buc, 1975) or even a four-state (Kastenschmidt et al., 1968; Busby & Radda, 1976) model of the allosteric transitions in phosphorylase *b* is undoubtedly an oversimplification if applied to the entire enzyme molecule. The proper alignment of catalytic groups, i.e., the transition into the "R" state, can apparently be achieved in more than one way, and in more than one step, just as inactive "T" conformations may be produced through several routes. The extremely complex machinery of the associating phosphorylase *b* dimer allows for this structural versatility.

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