Intersubunit Transmission of Ligand Effects in the Glycogen Phosphorylase *b* Dimer†

Martin K. Battersby‡ and George K. Radda*

ABSTRACT: The heterotropic interactions between AMP, the allosteric activator, and glucose 6-phosphate, an inhibitor, binding to glycogen phosphorylase *b* have been studied by a novel procedure. The affinity label 8-[*m*-(*m*-fluorosulfonylbenzamido)benzylthio]adenine has been shown to mimic the action of bound AMP when reacted with AMP binding sites on each monomer of the phosphorylase *b* dimer [Anderson, R. A., Parrish, R. F., & Graves, D. J. (1973) *Biochemistry 12*, 1895–1900]. The affinity label activates the enzyme and prevents AMP and glucose 6-phosphate binding, as probed by a spin-label and catalytic activity. A stable hybrid dimer, having only one monomer modified with the affinity label, has been isolated by using 5′-AMP–Sepharose affinity chromatography. The activity of the hybrid in the absence of AMP was a measure of the activity of the modified subunit only, while addition of AMP activated the unmodified subunit only. Glucose 6-phosphate binds only to the unmodified monomer, yet it inhibits the activity (in the absence of AMP) of the modified subunit, showing heterotropic interactions across the subunit interface. The dissociation constant for glucose 6-phosphate binding to the unmodified monomer was an order of magnitude higher than that for binding to native spin-labeled enzyme. This demonstrated that the affinity label modification also affects the unmodified subunit of the hybrid. Such interactions probably occur in vivo between AMP and glucose 6-phosphate binding to different subunits. Glucose 6-phosphate/AMP competition within one subunit was simply investigated by the use of the +AMP activity of the unmodified subunit of the hybrid which lacks the AMP homotropic binding properties of the native enzyme. The intrasubunit interaction was shown to be partially competitive.

The concept of allosteric transitions induced by an effector molecule resulting in an alteration of the properties of an enzyme was first introduced by Monod et al. (1963) and later extended to multisubunit enzymes (Monod et al., 1965). As an example of potential metabolic regulation by such an effector, the pioneer work of Cori et al. (1938) and Cori & Green (1943) was cited, demonstrating that activity could be induced in glycogen phosphorylase *b* (EC 2.4.1.1.) by AMP, which was not itself modified in the phosphorylase reaction.

The activity of phosphorylase *b* has been shown since to be regulated by many metabolites, of which AMP, the activator, and glucose 6-phosphate, an inhibitor, are particularly important (Fischer et al., 1971; Graves & Wang, 1972). Simulation of conditions in vivo (Morgan & Parmeggiani, 1964) indicated that, in addition to covalent activation by phosphorylation, regulation of phosphorylase *b* by allosteric effectors may be important in the control of glycogenolysis.

Recently, much interest has been focused on the transitions induced in the subunit adjacent to that which binds the effector or substrate. That such transitions occur has been demonstrated by Feldmann et al. (1976), who have prepared hybrid phosphorylase *b* dimers of inactive monomers, bearing pyridoxal 5′-phosphate analogues, and Sepharose-bound native monomers which, alone, are inactive. That this interaction can result in AMP-induced activity in the native monomers demonstrates that the correct conformation of the inactive subunit is essential to confer activity on the adjacent native subunit.

Morange et al. (1976) and Buc et al. (1973) showed that the extent of release of glucose 6-phosphate bound to phosphorylase *b* preceded the extent of AMP binding. It was suggested that the binding of one molecule of AMP to the enzyme results in the release of two glucose 6-phosphate molecules. This observation implies that the binding of AMP to one subunit alters the conformation of both subunits to a form which has a weak affinity for glucose 6-phosphate. It is possible that such intersubunit communication is important in the regulation of the enzyme in vivo.

In this paper, we describe the properties of a hybrid enzyme in which one of the subunits contains a covalent affinity label at the AMP binding site. Anderson et al. (1973) have shown that the enzyme modified with this reagent has similar properties to those of the enzyme in the presence of AMP. Here we demonstrate that the affinity of the unmodified subunit of the hybrid for glucose 6-phosphate is affected by the adjacent, modified subunit. In addition, the lack of homotropic effects in the hybrid means that the intrasubunit competition between AMP and glucose 6-phosphate on the unmodified monomer can be examined.

Materials and Methods

Phosphorylase *b* was prepared by the method of Fischer & Krebs (1962) with minor modifications for large-scale preparation. Cysteine was replaced by dithiothreitol in the recrystallization stages. Twice-recrystallized enzyme was stored at −20 °C as a freeze-dried powder obtained from a solution in 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, and 1 mM EDTA at pH 7.5. Enzyme solutions prepared from this powder had the same specific activity as the solution before freeze-drying. Before use the enzyme was recrystallized.

The enzyme activity was assayed in the direction of glycogen synthesis by estimation of the inorganic phosphate released by using the procedure of Hurst (1964), adapted for use on a Technicon Autoanalyzer. The assay mixture contained 16 mM glucose 1-phosphate, 1% oyster glycogen in 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, and 1 mM EDTA at pH 7.0. Small volumes of AMP and/or glucose 6-phosphate solutions were added where required. Such additions amounted to a change of no more than 5% in

† From the Department of Biochemistry, University of Oxford, Oxford OX1 3QU, England. *Received December 29, 1978; revised manuscript received May 22, 1979.* This work was supported by the Science Research Council and is a contribution from the Oxford Enzyme Group.

‡ Present address: Pharmaceutical Research Department, F. Hoffmann-La Roche and Co. Ltd., 4002 Basle, Switzerland.

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the total volume. Each assay was initiated by addition of the enzyme solution, suitably diluted where necessary, to the preincubated assay mixture at 30 °C. The unmodified native enzyme had specific activities within the range of 60–70 μmol of P_i min^{-1} mg^{-1} of protein at 30 °C in the presence of 1 mM AMP. Specific activities for the hybrid enzyme are expressed as means ± standard error of three determinations of activity of three different hybrid preparations.

The concentration of phosphorylase was determined by measuring the optical density at 280 nm by using $A_{1cm}^{0.1\%}$ = 1.32 (Kastenschmidt et al., 1968). All enzyme concentrations are expressed as monomer concentrations. A molecular weight of 100 000 per monomer was used (Cohen et al., 1971).

Triethanolamine hydrochloride, AMP, and glucose 1-phosphate were obtained from Boehringer Corp. Ltd. Tris, 2-mercaptoethanol, glucose 6-phosphate, and 2-glycerophosphate were purchased from Sigma Chemical Co. 5′-AMP–Sepharose was obtained from Pharmacia (Uppsala, Sweden). Oyster glycogen was obtained from British Drug Houses Ltd. [^{14}C]-8-[*m*-(*m*-Fluorosulfonylbenzamido)-benzylthio]adenine (FSA)[1] was kindly provided by Dr. D. J. Graves, Iowa State University, whose generosity is gratefully acknowledged. 4-(2-Iodoacetamido)-2,2,6,6-tetramethyl-piperidinyl-1-oxy spin-label was a Syva product. 4-Chloro-7-nitrobenzofurazan was purchased from Serva Feinbiochemica. All other chemicals were of the highest purity available from British Drug Houses Ltd. or Fisons Scientific Apparatus Ltd.

Modification of the AMP site of phosphorylase *b* was performed by the method of Anderson & Graves (1973) in 40 mM glycerophosphate, 2 mM EDTA buffer at pH 7.8 by addition of a 1.2–1.4 molar excess of FSA to a 20–25 μM enzyme solution. FSA was added as a small volume of a 2–3 mM stock solution in dimethyl sulfoxide. Incubation at 30 °C was for 1 h routinely, but for the preparation of hybrid mixtures 2 h was used. Following incubation, the enzyme was precipitated by addition of an equal volume of saturated ammonium sulfate solution, centrifuged, redissolved, and dialyzed against the appropriate buffer.

Concentration of modified enzyme was determined either by the method of Lowry et al. (1951) or from the optical density at 280 nm by using an extinction coefficient corrected for the small absorption of FSA. It was assumed for this that preparation of modified enzyme as described results in incorporation of 1 FSA molecule/monomer.

The preparation of spin-labeled phosphorylase *b*, the technique of ESR titrations, and the treatment of the results have been described previously (Campbell et al., 1972; Griffiths et al., 1976). Modification of phosphorylase *b* with 1 equiv of spin-label/monomer results in an 85:14 distribution of spin-label between the two rapidly reacting sulfhydryl groups (Griffiths et al., 1975). The ratio of the downfield to center resonance heights from the first derivative ESR spectrum (the ESR ratio) is used as an index of spin-label mobility (Campbell et al., 1972).

5′-AMP–Sepharose was swollen in 50 mM Tris, 10 mM 2-mercaptoethanol buffer at pH 8.2 and packed into a glass column of 9 mm internal diameter having a sintered glass gel support. The exact length of the bed will be given in the legend to the particular experiment. For routine hybrid purification, bed lengths of 100–110 mm were used. The buffer reservoir level was adjusted to give flow rates of 20–30 mL/h. The columns were run at room temperature. The optical density of the eluate was monitored at 280 nm in a SP 1800 Unicam spectrophotometer coupled to an AR25 chart recorder. A Hellma 10-mm path length quartz flow cell or a Unicam 2-mm path length quartz flow cell was used, depending on the amount of protein applied.

The radioactivity in the eluate was measured by collecting fractions directly into scintillation vials. The 0.8–1.0-mL fractions were taken up by addition of 15 mL of a scintillation fluid prepared with 250 mL of scintillation grade Triton X-100, 75 mL of AR toluene, 5 g of 2,5-diphenyloxazole (PPO), and 0.2 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP). The vials were maintained in a Beckman LS-233 scintillation counter without exposure to light for 1 h before counting began. Each vial was counted for 20 min, giving an accuracy of ±3% for peak fractions and ±5% for most remaining samples. Background counts were subtracted.

The reaction of 4-chloro-7-nitrobenzofurazan (NBf-Cl) with enzyme solutions was observed in the SP 1800 Unicam spectrophotometer at 420 nm. NBf-Cl was added to an ~10 μM enzyme solution as a 37.5 mM solution in ethanol. The reference cell contained buffer and an equivalent concentration of NBf-Cl. The number of reacted groups was calculated by using $\epsilon = 13.0 \times 10^6$ cm^2 mol^{-1} for the S-NBf derivative (Birkett et al., 1970). The reactions were carried out in 50 mM triethanolamine hydrochloride, 100 mM potassium chloride, and 1 mM EDTA buffer at pH 7.0 at 20 °C.

Ultracentrifuge experiments were performed on a Beckman Model E analytical ultracentrifuge at 20 °C. Sedimentation coefficients have not been corrected for viscosity, etc.

In all experiments involving the isolation of hybrid material, the dilute solutions eluting from the AMP–Sepharose columns were concentrated in "Minicon" B 15 concentrators (Amicon Corp.).

Results

(*A*) *Properties of the Modified Enzyme.* Anderson & Graves (1973) have shown that phosphorylase *b*, modified by reaction with a 1.2–1.4 molar excess of FSA, incorporates 1 FSA molecule/subunit. The modified enzyme showed, like phosphorylase *a*, an intrinsic catalytic activity equivalent to 24% of the activity of the unmodified enzyme in the presence of 1 mM AMP.

In our hands, the modified enzyme had a specific activity the same as that prepared by Anderson et al. (1973) (~9 μmol of P_i min^{-1} mg^{-1}). The +1 mM AMP specific activity of the initial enzyme before modification was higher than that used by Anderson et al. This may be the result of the different buffers used in the assay mixture.

The effect of ligand binding to spin-labeled phosphorylase *b* on the conformation of the enzyme can be monitored by the change in mobility of the spin-label (Campbell et al., 1972; Griffiths et al., 1976) which results in an alteration in the ESR spectrum. The ration of the downfield to center resonance heights (the ESR ratio) has been used as an index of spin-label mobility. Dissociation constants, calculated from the ESR ratio changes, for AMP and glucose 6-phosphate binding to spin-labeled phosphorylase *b* are 85 and 31 μM, respectively.

The effect of modification by FSA on the mobility of the spin-label is shown in Figure 1. The ESR ratio showed an initial fall, interpreted as being the result of binding, followed by a slow rise, due to the reaction. The ESR ratio of the product is higher than that of the unmodified enzyme, indicating increased spin-label mobility.

Unlike spin-labeled phosphorylase *b* (Griffiths et al., 1976), the ESR ratio of FSA-modified spin-labeled phosphorylase *b* (0.69) was not affected by concentrations of AMP or glucose

[1] Abbreviations used: FSA, 8-[*m*-(*m*-fluorosulfonylbenzamido)-benzylthio]adenine; NBf-Cl, 4-chloro-7-nitrobenzofurazan.

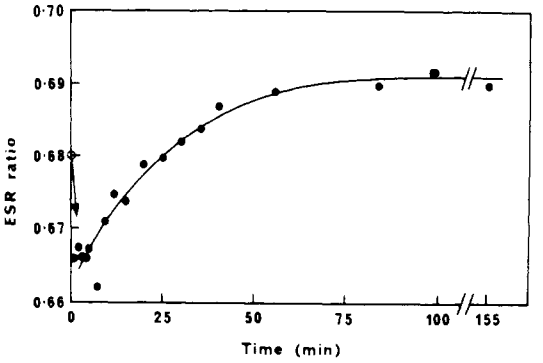

FIGURE 1: Effect of FSA modification on the ESR spectrum of spin-labeled phosphorylase *b*. The reaction of 52 μM spin-labeled phosphorylase *b* with a 1.2 molar excess of FSA in 50 mM Triethanolamine hydrochloride, 100 mM potassium chloride, and 1 mM EDTA buffer at pH 7.0, as monitored by the change in the ESR ratio. Control experiments showed that the reaction in this buffer is identical with that in glycerophosphate buffer. The observed ESR ratios have been normalized so that the initial ratio is 0.68 (Griffiths et al., 1976). FSA addition was at zero time.

6-phosphate up to 20 mM. It did, however, retain its sensitivity to glucose 1-phosphate and 2-glycerophosphate binding, giving ESR ratios of 0.67 and 0.64 in the presence of 100 mM glucose 1-phosphate and 40 mM 2-glycerophosphate, respectively. Campbell et al. (1972) have shown that such small changes in ESR ratio are highly reproducible.

In agreement with Anderson et al. (1973), the activity of FSA-modified enzyme was not affected by concentrations of glucose 6-phosphate up to 18 mM. However, high concentrations of AMP were found to *inhibit* FSA-modified enzyme activity. A double-reciprocal plot of this inhibition was linear (correlation coefficient = 0.998) with a *y*-axis intercept at 100% inhibition. The K_i derived from the *x*-axis intercept was 5.4 mM, which is consistent with AMP binding to the second low affinity AMP binding site on the enzyme monomer ($K_d \simeq 5$ mM, as measured calorimetrically; Ho & Wang, 1973). The observed inhibition is consistent with the observation that adenine and adenosine, which bind at this second site with higher affinity than to the allosteric site (Buc et al., 1974), also inhibit trace phosphorylase *b* activity in the absence of AMP (Morange et al., 1976).

The ESR and activity results indicate that FSA behaves like AMP, preventing glucose 6-phosphate binding to phosphorylase *b* (Wang et al., 1970). It also prevents AMP interaction at the high affinity allosteric site.

The similarity between modified enzyme and native enzyme in the presence of AMP was further demonstrated in a study of the sulfhydryl group reactions. It has been shown (Birkett et al., 1971) that the reaction of phosphorylase *b* with NBf-Cl is biphasic. The set of slowly reacting groups can be partially protected by saturating concentrations of AMP. To compare the protective ability of AMP and FSA, NBf-Cl labeling experiments were carried out under identical conditions. The time courses of the reactions are shown in Figure 2. The FSA modifying reagent is better at protecting the slowly reacting groups than AMP. The rate constants, calculated by using the method of Freedman & Radda (1968), assuming an end point of four groups per monomer (Avramovic-Zikic et al., 1970), are given in Table I.

It is known that the rate of inactivation of phosphorylase *b* by NBf-Cl is reduced by AMP (Birkett et al., 1971). Figure 3 demonstrates that modified enzyme has the same inactivation time course as native enzyme in the presence of 5 mM AMP. In the absence of peptide mapping experiments, the interpretation of these results is difficult. FSA appears to protect

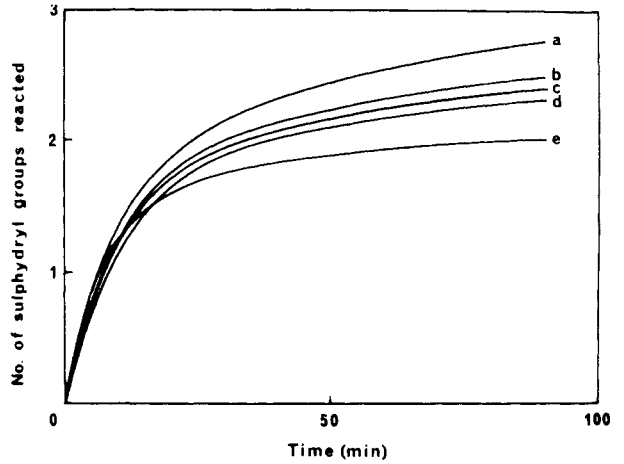

FIGURE 2: 4-Chloro-7-nitrobenzofurazan reactions. The reactions of 10 μM native phosphorylase *b* with 375 μM 4-chloro-7-nitrobenzofurazan in the absence (a) and the presence of 1 (b), 2 (c), and 5 mM (d) AMP. The reaction of 10 μM FSA-modified enzyme in the absence of AMP (e) is unaffected by addition of 5 mM AMP. The buffer was 50 mM triethanolamine hydrochloride, 10 mM potassium chloride, and 1 mM EDTA at pH 7.0. The temperature was 20 °C.

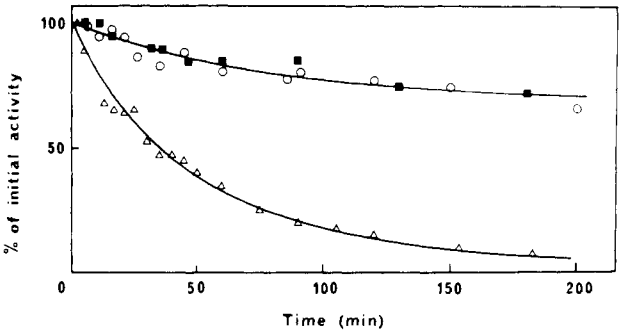

FIGURE 3: Inactivation by 4-chloro-7-nitrobenzofurazan. The time courses of inactivation of 9 μM concentrations of FSA-modified phosphorylase *b* (■), native phosphorylase *b* + 5 mM AMP (○), and native phosphorylase *b* (△) by 375 μM 4-chloro-7-nitrobenzofurazan at 20 °C. Each point on the native phosphorylase *b* curve represents an average from two identical reactions, while the other points are from one reaction of each.

Table I: Rate Constants for the NBf-Cl Reactions of Figure 2[a]

form of enzyme used ± AMP	fast kinetic set: no. of groups	fast kinetic set: k (M^{-1} min^{-1})	slow kinetic set: no. of groups	slow kinetic set: k (M^{-1} min^{-1})
native phosphorylase *b*	1.8 (2)	315	2.2 (2)	16.1
FSA-phosphorylase *b*	1.59 (2)	398	2.4 (2)	5.6
native + 5 mM AMP	1.74 (2)	271	2.26 (2)	8.7
native + 2 mM AMP	1.77 (2)	284	2.23 (2)	10.1
native + 1 mM AMP	1.8 (2)	292	2.2 (2)	11.1

[a] The rate constants were derived by the method of Freedman & Radda (1968). An end point of four groups is assumed. The calculated number of groups in each kinetic set is never integral because of other much slower reactions, denaturation, and air oxidation of sulfhydryl groups prior to the experiment. Our estimates of the number of groups in each set are given in parentheses.

the sulfhydryl group responsible for inactivation as efficiently as 5 mM AMP, but it is more efficient than AMP at protecting the other slowly reacting group (Figure 2).

The effect of the reagent on the quaternary structure of the enzyme is also similar to that of AMP. Anderson et al. (1973) have reported that, at 12.8 °C, the modified enzyme tetramerizes ($s_{20,w} = 12.7$) in the same way that unmodified phosphorylase *b* does, at the same temperature, in the presence

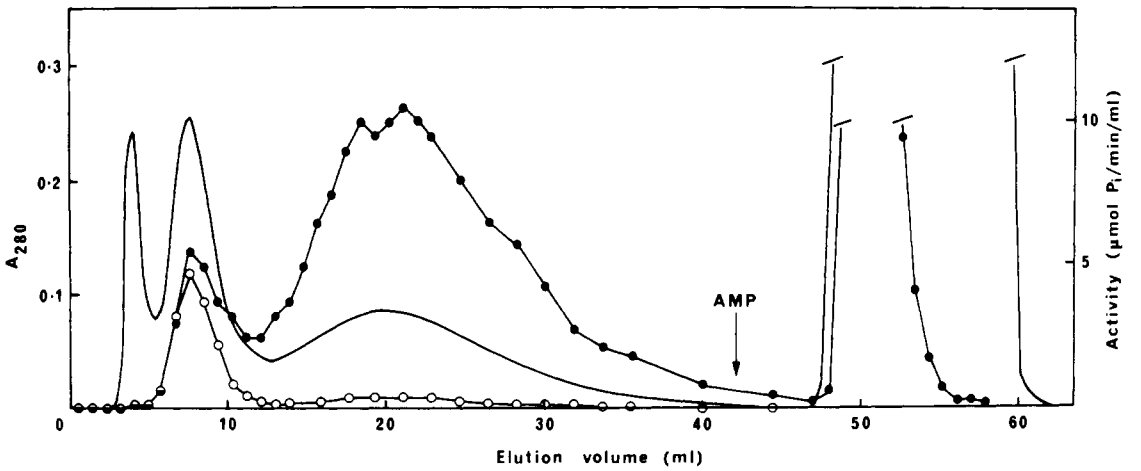

FIGURE 4: Affinity chromatography of a half-reacted mixture. Phosphorylase *b* (25 mg) was reacted with 0.5 equiv of FSA. The dialyzed sample was applied to a 0.9 × 8 cm AMP–Sepharose column in 950 mL. Optical density (smooth curve) was monitored with a 2-mm flow cell at 280 nm. The sample was applied at 0-mL elution volume and followed with the starting buffer of 50 mM Tris-HCl, 10 mM 2-mercaptoethanol at pH 8.2. AMP (20 mM) in this buffer was used to elute retained enzyme. Fractions were assayed at 30 °C for +1 mM AMP (or + eluted AMP) (●) and −AMP (○).

of 1 mM AMP. We have extended this study by observing the sedimentation properties of the enzyme at 20 °C in a 400 mM imidazole citrate buffer at pH 6.35.

It is known (Hedrick et al., 1969) that native phosphorylase *b* monomerizes (s = 4.7) in this buffer, but this effect is reversed by addition of AMP, forming mainly tetramer (s = 11.6). Modified enzyme in this buffer also sediments in the tetramer form (s = 12.2) and also as higher aggregates (s = 17.6), while the presence of monomer could not be detected. Both modified and unmodified enzymes sediment as dimers in a nondeforming buffer (s = 8.8 and 8.9, respectively, in 50 mM Tris and 10 mM 2-mercaptoethanol at pH 8.2). We conclude that FSA, like AMP, opposes the "deforming" and monomerizing action of imidazole citrate.

(B) Preparation of a Hybrid Dimer. Previous preparations of phosphorylase *b* hybrid dimers containing different pyridoxal derivatives utilized monomer formation in imidazole citrate or on reaction with excess *p*-hydroxymercuribenzoate. The FSA-modified enzyme is not monomerized in this way, and when reacted with a 26-fold excess of *p*-(chloromercuri)-phenylsulfonic acid, it still sedimented as a dimer (s = 10.0).

We have therefore developed a new approach. Reaction of phosphorylase *b* with 0.5 equiv of FSA yielded a mixture of fully labeled dimers, hybrid dimers, and unmodified dimers. These species were separated on an AMP–Sepharose affinity column. This method is similar to that of Danchin & Buc (1973) but was on a larger scale and avoided the use of stepwise elution. A 50 mM Tris and 10 mM 2-mercapto-ethanol buffer at pH 8.2 was similarly used to prevent monomerization within the column which might take place at lower pH (Davis et al., 1967). Control experiments showed that unmodified enzyme was retained on the gel, with some leakage, in agreement with Sørensen & Wang (1975). Fully modified dimers passed through the column unretarded.

The elution profile of phosphorylase *b* reacted with 0.5 equiv of FSA is shown in Figure 4. The absence of activity in the first peak showed it to be an impurity excluded from the gel. The second peak showed the same activity in the presence and absence of AMP and corresponded to the unretarded fully modified dimers. The considerably retarded peak (max ~20 mL) showed AMP-stimulated and intrinsic activity, as might be expected of a hybrid dimer of one FSA-modified and one unmodified subunit. Analysis of the radioactivity in fractions from affinity chromatography of a larger sample (Figure 5)

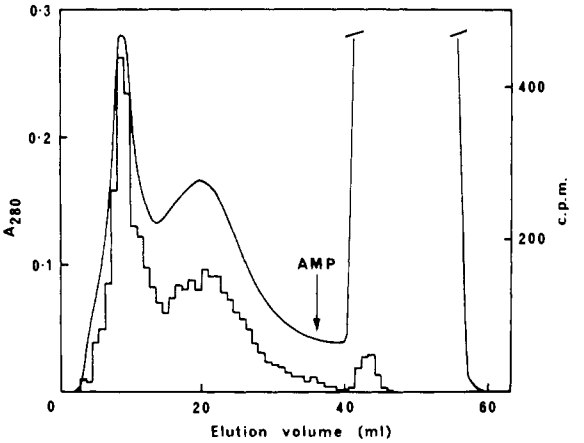

FIGURE 5: Radioactivity content of hybrid. Approximately 40 mg of the half-reacted FSA-modified phosphorylase *b* mixture was applied in 500 μL to a 0.9 × 11 cm AMP–Sepharose column. Optical density at 280 nm (smooth curve) was monitored by using a 2-mm flow cell. Radioactivities were counted for 20 min. Other conditions are as described in Figure 4.

showed that the retarded species was less heavily labeled than the fully modified enzyme. Calculating the ratios of counts per minute to average optical density of the two peak fractions of the fully modified enzyme and normalizing them to 1, we obtained a value of 0.6 for the normalized cpm/OD ratio of the four peak fractions of the retarded peak.

Pooled concentrated fractions obtained from the retarded peak were also treated with an excess of modifying reagent at 30 °C for 1 h. The product eluted from an AMP–Sepharose column in the same elution volume as the fully modified enzyme. This provides further evidence that the retarded species is a hybrid of one modified and one unmodified subunit.

To ensure that highly purified hybrid was used in our activity measurements, the material from the center of the hybrid peak was pooled and reapplied to a second washed column. Pooled concentrated fractions from the eluted hybrid peak were then applied to a third column. The hybrid obtained from this column was then used for the experiments.

(C) Properties of the Hybrid Enzyme. Before any studies were done, it was essential to determine the stability of the hybrid. A sample of hybrid, partially purified by two passages through the affinity column, was taken and concentrated to 107 μM. This sample was maintained at 4 °C in 50 mM Tris,

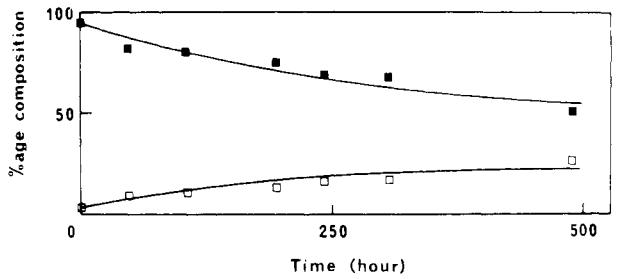

FIGURE 6: Time course of disproportionation at 4 °C. Percentage composition was calculated from the ratios of peak areas from elution profiles of sample aliquots. The preparation and treatment of the sample is described in the text. (■) Disproportionation of the hybrid species with concomitant formation of the homologous dimers; (□) formation of one homologous dimer species only.

10 mM 2-mercaptoethanol buffer at pH 8.2, and, during the following 20 days a small aliquot was removed, from time to time, and applied to a washed affinity column. Because of the small volume applied, resolution of the fully modified and hybrid peaks was good and the proportions of the two species could easily be estimated from the areas under the respective peaks. During storage a slow reduction in the proportion of hybrid, and a corresponding increase in the fully modified species, was observed. The time course of the disproportionation is shown in Figure 6. While the amount of unmodified enzyme retained on the column was not measured, we assumed that it was formed at the same rate as the fully modified enzyme. The results are therefore displayed to indicate the changes in all three species.

The observation that fully modified enzyme can be formed from hybrid enzyme confirms that the material isolated from the half-reacted mixture is the hybrid. In addition, disproportionation is slow enough to study the properties of the hybrid. All subsequent experiments were done within 12 h of isolation of the hybrid, and we can safely assume that changes in composition during this period are negligible.

At 20 °C, the pure hybrid showed a single peak in the anlytical ultracentifuge (s = 8.87). The sedimentation coefficients of modified and unmodified dimers in the same buffer were 8.8 and 8.9, respectively. No monomer or tetramer species were detected in the hybrid sample.

To determine the effect of the modified subunit on the unmodified one, we studied the activation by AMP and inhibition by glucose 6-phosphate of the hybrid. In the absence of AMP, the hybrid showed a specific activity (1.68 ± 0.29 μmol of P_i min^{-1} mg^{-1}) less than half that of the fully modified enzyme (9–12 μmol of P_i min^{-1} mg^{-1}). This implies that the inactive unmodified monomer of the hybrid inhibits the intrinsic activity of the modified monomer. In the presence of AMP, the hybrid showed approximately half (30.4 ± 2.5 μmol of P_i min^{-1} mg^{-1}) the activity of native enzyme (60–65 μmol of P_i min^{-1} mg^{-1}), indicating that the FSA-modified monomer has little influence on the activity of the unmodified monomer.

It is reasonable to assume that the −AMP activity of the hybrid is due only to the activity of the modified subunit and that the +AMP activity is a measure of the activity of the unmodified subunit. Our measurements are unable, however, to exclude the possibility that the affinity label confers activity on the unmodified subunit of the hybrid, nor can we exclude the possibility that AMP binding to the unmodified subunit also activates the modified subunit. We believe that our interpretation is the most reasonable in view of the observations of Feldman et al. (1976), who have shown that inactive phosphorylase b monomers carrying pyridoxal phosphate analogues allow potentially active monomers of a hybrid to be nearly fully activated by AMP. This result is analogous to our demonstration that, in the presence of AMP, the activity of the hybrid can be accounted for by assuming that the unmodified monomer is nearly fully activated. Moreover, if AMP binding to, or affinity labeling of, one subunit resulted in activation of both subunits, then one might expect to be able to demonstrate, in the native enzyme titrated with AMP, that the extent of increase in activity preceded the extent of AMP binding. Morange et al. (1976) have compared the concentration of AMP and analogues required to half-activate phosphorylase b (K_a) with that required to half-saturate the enzyme ($K_{0.5}$). For AMP, these two values are similar (K_a = 11 μM and $K_{0.5}$ = 16 μM at 4 °C). We therefore suggest that neither AMP nor the affinity label at the nucleotide binding site can induce *activity* in the adjacent subunit.

Since the fully modified enzyme is not inhibited by glucose 6-phosphate [under section A above and Anderson et al. (1973)] and no glucose 6-phosphate binding was detected by ESR, we may assume that any inhibition of the hybrid species by glucose 6-phosphate is a result of interaction with the unmodified subunit.

The −AMP activity of the hybrid, due to the modified subunit, is inhibited by glucose 6-phosphate binding to the unmodified monomer. A double-reciprocal plot of 1/% inhibition vs. 1/[Glc 6-P] was linear and yielded a K_i of 588 ± 49 μM from three different hybrid experiments. This is an order of magnitude higher than the K_d for glucose 6-phosphate binding to spin-labeled phosphorylase b (31 μM; Griffiths et al., 1976). It should be pointed out that it is valid, in this case, to compare K_i values obtained kinetically with dissociation constants measured using a spin-label. A close parallel exists between the binding of AMP to spin-labeled enzyme as detected by ESR in the absence of substrates (glycogen and glucose 1-phosphate) and the activity measured in their presence (Campbell et al., 1972). In addition, glycogen has no effect on the affinity of native enzyme for glucose 6-phosphate (Griffiths et al., 1976), and although glucose 1-phosphate causes a slight reduction of enzyme affinity for glucose 6-phosphate (Griffiths, 1974), this could only account for a small fraction of the 20-fold increase in K_d observed here. We conclude that the affinity label has a marked effect on the affinity of the unmodified subunit of the hybrid for glucose 6-phosphate.

Double-reciprocal plots demonstrating AMP activation of the unmodified subunit of the hybrid in the presence and absence of fixed glucose 6-phosphate concentrations are shown in Figure 7. The method of computer generation of the lines is described under the Discussion. Visual inspection of the points alone shows that the plots are linear, providing additional evidence that the hybrid differs from the native enzyme for which similar plots show deviations from linearity resulting from the cooperative binding of AMP. Analysis of AMP activation of native enzyme, under the same assay conditions as those used in Figure 7, using Hill plots showed that the Hill coefficients (n) were 1.43 and 1.53 in the presence of 0 and 500 μM concentrations of glucose 6-phosphate, respectively.

The K for AMP activation of the hybrid in the absence of glucose 6-phosphate is 86 ± 5 μM. This is the same as the value obtained for AMP binding to spin-labeled enzyme assuming no cooperativity (Griffiths et al., 1976).

Discussion

We have shown that a stable hybrid dimer of phosphorylase b can be isolated. The FSA-modified monomer is in a state similar to that occurring when AMP binds. However, the adjacent unmodified monomer does not adopt the same state

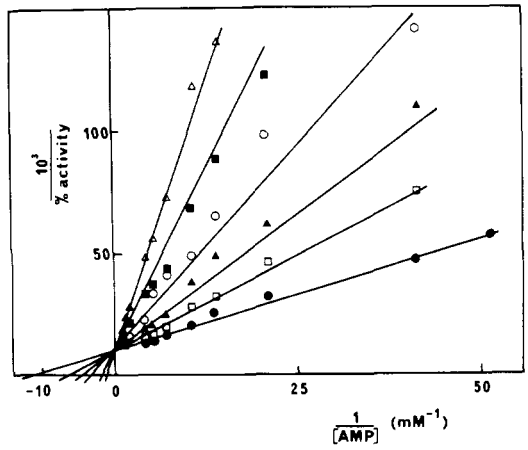

FIGURE 7: AMP activation of hybrid in the presence and absence of glucose 6-phosphate. A double-reciprocal plot from assays of hybrid enzyme at 30 °C. Activities are expressed as a percentage of the activity measured in the presence of 1 mM AMP and in the absence of AMP. Low basal activity due to the FSA-modified monomer was measured at each glucose 6-phosphate concentration in the absence of AMP and subtracted. Fixed concentrations of glucose 6-phosphate were 0 (●), 0.5 (□), 1 (▲), 2 (○), 5 (■), and 10 mM (△). The solid lines are computer simulation plots using the method described in the text. The value of K_a' has been adjusted to give the best visual fit to the experimental data. The values of constants used in the simulation were K_a = 90 μM, K_i = 600 μM, K_a' = 1.9 mM, K_i' = 12.7 mM, V_{max} = 100%, and x = 1.

as that of the modified subunit, as might be expected if the symmetrical model of Monod et al. (1965) was applicable to the transitions of phosphorylase *b*. Each monomer affects the other, but neither can constrain the other into its own state. Guênard et al. (1977) came to the same conclusion in their study of a hybrid of brain and muscle phosphorylase *b* isoenzymes.

We have shown (a) that glucose 6-phosphate binding to the unmodified subunit can affect the activity of the adjacent modified subunit and (b) that the FSA-modified subunit reduces the affinity of the unmodified subunit for glucose 6-phosphate. This is a demonstration that heterotropic interactions occur across the subunit interface. It provides confirmation, by a direct method, that the binding of one ligand to a dimer can release the other ligand from its site on the adjacent monomer, as suggested by Morange et al. (1976).

This could account for the discrepancy between phosphorylase *b* activity observed in resting muscle and that calculated from the known metabolite concentrations (Fischer et al., 1971). The major inhibitor of phosphorylase *b*, glucose 6-phosphate, binds to ~65% of the phosphorylase *b* in muscle (Busby & Radda, 1976), but because some will be bound in a hybrid form (i.e., 1 glucose 6-phosphate molecule/dimer), a greater percentage of the enzyme will be inaccessible to AMP as a result of the intersubunit heterotropic interaction. Until now such an effect could not be taken into account when calculating phosphorylase *b* activities.

The lack of homotropic interactions for AMP binding to the hybrid means that competition between AMP and glucose 6-phosphate is simply studied. Lines drawn through the experimental points of Figure 7 for the best visual fit intersected at V_{max}, but a plot of the slopes of these lines vs. glucose 6-phosphate concentration indicated that the inhibition is "hyperbolic". This is the result of "partial competition" between the ligands, as indicated by the results of Wang et al. (1970) in their study of glutaraldehyde-modified phosphorylase *b*.

The scheme for partial competitive inhibition for our system is given by a modification of the Botts & Morales scheme

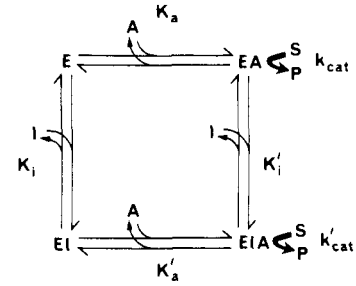

FIGURE 8: Scheme for partial competitive inhibition. It is assumed that the activator, AMP (A), and the inhibitor, glucose 6-phosphate (I), bind to the enzyme (E) at different sites. We also assume that both the enzyme–AMP complex (EA) and the ternary complex (EIA) are able to convert substrates (S) to products (P) with catalytic constants k_{cat} and k_{cat}', respectively. K_a, K_i, etc., refer to the equilibrium constants for ligand binding.

(1953) as in Figure 8. The rate equation is given by eq 1, where $x = k_{cat}'/k_{cat}$ and $V_{max} = k_{cat}[E_0]$, $[E_0]$ being the total enzyme concentration.

$$V = \frac{[A](V_{max}K_i' + xV_{max}[I])/(K_i' + [I])}{(K_aK_i' + [I]K_a')/(K_i' + [I]) + [A]} \quad (1)$$

This equation predicts hyperbolic inhibition of the type observed in our experiments. Since V_{max} is independent of glucose 6-phosphate concentration (Figure 8), the expression for V_{max}^{app} in eq 1, i.e., $V_{max}^{app} = (V_{max}K_i' + xV_{max}[I])/(K_i' + [I])$, should also be independent of [I]. For this to be the case, the value of x, the ratio of the catalytic activities of EA and EIA, must be 1, indicating that the activity of the ternary complex is the same as that of the enzyme–AMP complex.

The values of K_a and K_i are known to be 90 and 600 μM, respectively (see Results, section C). These values can be substituted into eq 1, together with the known values of V_{max} and x = 1.

The lines shown in Figure 7 were computer generated by using the constants described above and an extimated value of K_a', which also defined the value of K_i'. All of the lines were generated by using the single set of constants given in the legend to Figure 7, the value of K_a' being adjusted to provide a good visual fit with all of the experimental points of Figure 7.

The close agreement between the calculated and experimental data indicates that the scheme in Figure 8 is a valid description of the intrasubunit competition between AMP and glucose 6-phosphate.

As has been mentioned, there are a number of indications that the FSA affinity label is not able to influence the enzyme in an exactly equivalent manner to AMP, and recent reports of alternative potential reagents suggest the possibility of a similar study using reagents behaving more like AMP.

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Protected Nucleotide Sequences in Nuclear Ribonucleoprotein†

Leonard H. Augenlicht

ABSTRACT: The rapidly labeled nuclear ribonucleic acid in human carcinoma cells which is protected by protein from digestion by staphylococcal nuclease (EC 3.1.4.7) has been investigated. A simple and discrete sequence specificity was not found, but the protected RNA fragments are rich in G + C and were shown by fingerprinting to comprise a nonrandom subset of all heterogeneous nuclear ribonucleic acid (hnRNA) sequences enriched in the sequences AGC, GGC, AGGC, and GAGC. There was no detectable enrichment for double-stranded RNA in the protected fraction. These data provide the first evidence that the association of any protein with hnRNA is nonrandom with respect to nucleotide sequence.

The most direct evidence that eucaryotic nRNA is complexed with protein in situ is electron microscopic work on the nuclei of a variety of cell types. RNA in ribonucleoprotein (RNP) associated with regions of chromatin active in transcription has been documented in the lampbrush chromosomes of amphibian oocytes (Gall & Callan, 1962; Snow & Callan, 1969; Miller & Hamkalo, 1972; Miller & Bakken, 1972), in the puffs of insect polytene chromosomes (Beermann & Bahr, 1954; Swift, 1959; Stevens & Swift, 1966), and in mammalian cells (Miller & Bakken, 1972). In fact, more recent evidence (Miller & Hamkalo, 1972; Miller & Bakken, 1972; McKnight & Miller, 1976; Malcolm & Sommerville, 1974) indicates that RNA is probably complexed with protein almost immediately upon synthesis, before completion of the polynucleotide. RNA as RNP has also been clearly demonstrated in the nucleus free of chromatin (Beermann & Bahr, 1954; Swift, 1959; Stevens & Swift, 1966; Monneron & Bernhard, 1969).

Several methods of biochemical preparation of nuclear ribonucleoprotein (nRNP)[1] have been reported. RNP particles of 30–40 S can be extracted from intact nuclei (Samarina et al., 1968). These particles generally consist of a limited

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[1] Abbreviations used: nRNP, nuclear ribonucleoprotein; EDTA, ethylenediaminetetraacetate; $NaDodSO_4$, sodium dodecyl sulfate; MEM, minimum Eagle's medium; dsRNA, double-stranded RNA.

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