

Affinity Labeling of the Adenosine 5'-Monophosphate Binding Site of Rabbit Muscle Glycogen Phosphorylase *b* with an Adenosine 5'-Monophosphate-Cobalt(III) Complex*

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SUMMARY

A new affinity labeling method is presented which may be used for enzyme-catalyzing reactions in the presence of magnesium. This method uses the substrate-cobalt(III) analog of the substrate-magnesium complex as a covalent label of the active site. The specific case of rabbit muscle glycogen phosphorylase *b*, which has been covalently labeled with a AMP-Co(III) derivative at the allosteric site, is discussed. The stoichiometry and the extinction coefficient of the label have been determined. The association of this cobaltic complex to the enzyme is stable. The label destroys the activity on the subunit where it is bound; it slightly affects the binding of orthophosphate and it changes the response to the glycogen substrate from a noncooperative pattern to a cooperative binding. This shows that the label has an effect on the quaternary structure of phosphorylase *b*. In addition to the covalent binding of the label to the allosteric site, it is shown that its association can be reversed at will, i.e. it can be removed by the addition of small molecules containing thiol groups.

redox potential, they are frequently stable because their ligands have a low probability of being displaced in solution by an external competitor (5). It is therefore possible to prepare stable effector-Co(III) complexes by electrolytic oxidation, provided that the liganding groups are stable under these conditions.

We thought that the entropy fraction of the free energy barrier for substitution could be largely decreased once the Co(III)-effector complex is properly positioned in an enzymatic rigid site. There, at least one ligand (for example a carbonyl group of the protein which usually stabilizes the corresponding Mg^{2+} -effector complex) should be able to substitute for a nonessential ligand of the cobaltic complex (e.g. a water molecule or a Cl^- anion) and thus lead to a stable protein-Co(III)-effector ternary complex.

We have synthesized a 5'-AMP-Co(III)¹ derivative and determined its stoichiometry and extinction coefficient. Its special configuration has not yet been obtained. We have, however, shown the specificity of the binding of this complex with rabbit muscle glycogen phosphorylase *b* (α -1,4-glucan-orthophosphate glucosyltransferase, EC 2.4.1.1.). The association of this cobaltic complex to the enzyme is stable; however, 5'-AMP and cobalt can be displaced at will by addition of thiol derivatives, such as 2-mercaptoethanol. We have shown that the association occurs at the 5'-AMP binding site and that this binding changes the catalytic activity of the enzyme. Such affinity labeling provides a unique tool to investigate the structure of hybrid dimers, one subunit of which is labeled with 5'-AMP-Co(III). These hybrids allow us to study the interactions among the different substrates and to propose a pattern of relationship between several sites of phosphorylase *b*.

EXPERIMENTAL PROCEDURE

Glycogen Phosphorylase—Phosphorylase *b* was prepared as described by Krebs *et al.* (6). Its specific activity at 28° in a medium containing 2.5 mg of glycogen per g of H_2O , 1 mM 5'-AMP, and 12 mM orthophosphate (pH 7.0) was $125 \pm 5 A_{340nm}$ per min in a Tris-acetate buffer (pH 7.0, 50 mM) (7).

The standard medium for the conservation of phosphorylase *b* crystals contained 1 mM 5'-AMP, 10 mM $MgCl_2$, 50 mM 2-mercaptoethanol, and 50 mM Tris-acetate, pH 7.0.

5'-AMP-Co(III) Synthesis—5'-AMP-Co(III) was prepared by

¹ The abbreviations used are: AMP-Co(III), octahedral complex of AMP and Co^{3+} ; DTT, dithiothreitol.

About one-third of the currently purified enzymes require a magnesium divalent cation as a necessary cofactor or activator (1). Physicochemical studies have provided some information about the local structure of the protein around this ion but it is apparent that detailed structural information requires a method of specifically labeling the amino acids at these sites.

It is known that the cobaltic Co(III) cation, like magnesium, binds most of its ligands in an octahedral ligand field. The distances between cobalt and nitrogen or cobalt and oxygen are very similar to those observed in the corresponding magnesium complexes because these atoms have the same ionic radius (0.065 nm) (2-4). Although the Co(III) complexes often have a high

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electrolytic oxidation from a 100-ml solution of 5'-AMP (10 mM), CoCl₂ (11 mM), and KCl (50 mM) brought to pH 6.5 with KOH. The reagents were of the highest available grade. A platinum anode (alloyed with 10% iridium) rotating at about 500 rpm was immersed into the reaction solution and was connected to the cathode solution (saturated KCl, pH 0) by an agar bridge saturated in KCl. The rotating anode ensures proper stirring of the solution. A constant voltage of 1.1 volt was maintained for 48 to 60 hours between the two electrodes. The temperature was held constant at 20° and in some experiments at 4°. pH was maintained in the anodic solution in the range of 4.0 to 6.5 with dilute KOH. The anodic solution became strongly colored (yellow at pH 4, dark blue at pH 6.5), and was brought to pH 4 with HCl and concentrated to 5 ml in a rotating evaporator. This solution was then passed on a Bio-Gel P-2 column (100 × 2 cm) and eluted in KCl (1 mM) at pH 6.5. The first minor peak to be eluted (A) was olive green at pH 6.5. The second one (B) was dark violet. The 5'-AMP peak, partially overlapping with the Co²⁺ peak, was eluted just after Peak B. The tail of Peak B slightly overlapped with 5'-AMP (column effluent was monitored at 260 nm for AMP and at 550 nm for cobalt ions).

The yield after 60 hours of electrophoresis was about 10%. The recovery after the Bio-Gel P-2 column was usually 60% (for Peak B).

Characterization of Products—On high voltage electrophoresis on Whatman 3MM paper in potassium acetate (50 mM, pH 3.5), B migrates as a single band, negatively charged with $R_F = 0.43$ (taking the R_F of the Co²⁺ ion as -1.00), well separated from AMP ($R_F = +0.05$).

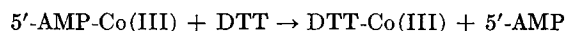
The relative amount of AMP and cobalt in complex B was obtained by three different methods: (a) double labeling, (b) simple labeling, and (c) spectrophotometry.

1. A preliminary characterization was obtained using a double labeling of the complexes with [¹⁴C]AMP (2 μCi per mM) and ⁵⁸Co (10 μCi per mM). After elution from the P-2 column the doubly labeled compounds were counted in 10 ml of Bray's scintillation solution (8) in a SL40 Intertechnique counter. The precision of the counting is poor because of interferences between β-rays of ¹⁴C and scintillation induced by ⁵⁸Co decay. However, this measurement indicates that Fraction A contains roughly 2 AMP molecules per Co(III) ion, whereas Fraction B contains an equal amount of both. The results are presented in Table 1 (first line): B is a 1:1 AMP-Co complex.

2. To avoid the interference between ⁵⁸Co and ¹⁴C, Product B was prepared from nonradioactive cobalt and [¹⁴C]AMP and

purified on Bio-Gel P-2. The AMP content was obtained from samples counted in Bray's scintillation solution. This method allows a direct evaluation of the absorbancy per AMP molecule at 260 nm for Complex B ($\epsilon = 22,000$). The cobalt content was obtained from an independent method. The cobalt assay is as follows: the complex is destroyed at alkaline pH by small molecules containing thiol groups (9). This thiolysis gives rise to an orange complex whose absorbancy is characteristic for cobalt at 480 nm. At pH 7.5, a solution containing 10 to 50 mM dithiothreitol or 2-mercaptoethanol reacts quantitatively with a Co²⁺ solution of known molarity in the presence of oxygen, to give an orange Co(III) complex which has an extinction coefficient of 9000 per equivalent of cobalt at 480 nm. Absorbancy follows Beer's law for solutions less concentrated than 50 μM in cobalt. Well characterized complexes such as EDTA-Co(III) gives the same thio complex, with the same absorbancy (but at a slower rate). In the absence of oxygen, Co²⁺ does not give the orange complex but a light green one, whereas EDTA yields the orange complex. The electron spin resonance of a DTT-cobalt complex (1 mM) has been investigated at a variable magnetic field in the X band. No signal is observed over a wide range of power and modulation: the complex is a low spin cobaltic one, even though thiol molecules are reducing agents.

The reaction which takes place is therefore:



From the absorbancy at 480 nm of the DTT-Co(III) complex, the initial amount of Co(III) in Fraction B is obtained and compared to the AMP content measured by scintillation counting. The result, shown in Table I, Line 2, shows a 1:1 stoichiometry of AMP *versus* cobalt.

3. The spectrophotometric determination of the cobalt content of a sample of Complex B enables one to subtract differentially the contribution of DTT-Co(III) absorbancy at 260 nm (see legend, Table 1) and thus to measure the AMP content of the thiolized complex (using $\epsilon = 15,400$ for AMP). One also obtains a 1:1 stoichiometry in this case (Table I, Line 3).

The thiolically displaced complex has been further characterized by chromatography on PEI-cellulose plates: one observes, for two LiCl concentrations (0.5 M and 1 M), that AMP is recovered. Electrolysis has therefore not altered the AMP molecule.

The molecular weight of the complex has been obtained using a calibrated Bio-Gel P-2 column (Fig. 1); it yields a value of about 510. This value is consistent with a complex containing

TABLE I
Determination of the stoichiometry of cobaltic complexes

Method	Complex A			Complex B			Phosphorylase <i>b</i> -AMP-Co(III)		
	Co	AMP	AMP-Co ratio	Co	AMP	AMP-Co ratio	Co	AMP	Phosphorylase <i>b</i>
Double labeling ^a (mM)	0.08	0.21	2.65:1	0.86	0.82	1.05:1			
Simple labeling (mM)				0.63	0.64	0.98:1			
Absorbancies ^b (μM)	12.2	24.3	1.99:1	27.7	27.9	1.01:1	6.4	6.0	6.1

^a Based on the radioactivity of a doubly labeled complex (see text) [¹⁴C]AMP (2 μCi per mM)-⁵⁸Co (10 μCi per mM).

^b The concentration of the components was determined spectroscopically after dissociation of the Co(III)-AMP complex with DTT (see text). The following extinction coefficients were used: Co(III)-DTT, $\epsilon_{480} = 9,000$; AMP, $\epsilon_{260} = 15,400$; phosphorylase *b*, $\epsilon_{280}^{1\%} = 13.2$. Phosphorylase *b* is labeled with Complex B. For

each of the two AMP-Co(III) complexes, a stock solution of known absorbancy at 260 nm was added to a 10-mm solution of DTT in Tris buffer (pH 7.5) and read after evaluation of the Co(III) content at 480 nm against the same DTT solution at the same final concentration in cobalt, in the presence of oxygen (thus containing the same amount of DTT-Co(III)).

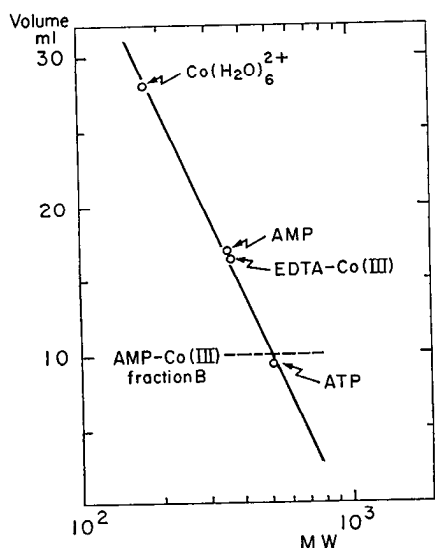


FIG. 1. Elution of various nucleotides and cobalt complexes on a Bio-Gel P-2 column in 1.25 M KCl. As the molecular weights of these derivatives are known except for the B complex, this allows evaluation of its molecular weight: one finds 510 ± 20 , a value consistent with the formula: $(\text{AMP}, 2 \text{ Cl}^-, \text{H}_2\text{O}) [\text{Co}(\text{III})]$.

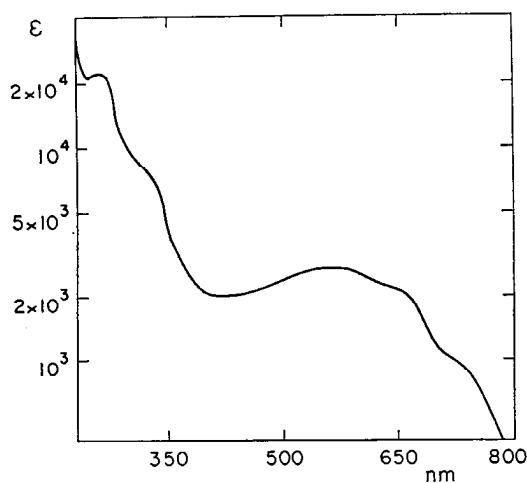


FIG. 2. Absorption spectrum of AMP-Co(III) in the near ultraviolet and the visible. The spectrum is recorded in a Cary model 14 spectrophotometer. The buffer was Tris-acetate (pH 6.9, 0.05 M).

one AMP and one cobalt. The precise stereochemical characterization of Complex B is currently under investigation.

Fig. 2 shows its absorption spectrum at pH 6.9 with a maximum at 260 nm ($\epsilon = 22,000$) and a broad maximum at 560 nm ($\epsilon = 2,800$). The absorption bands in the visible region are due to $d-d$ electronic transitions of the cobalt ion. The shoulder in the near ultraviolet region is due to electron transfer from charged ligands to the central ion. The intense near ultraviolet bands can be interpreted as electron transfer absorption bands from the ligands, which, in addition of 5'-AMP may be 2 or 3 Cl^- ions. By comparison with known cobaltic complexes (10), the 5'-AMP derivative may be tentatively described as a *cis*-dichloro complex, although the likely contribution of the $-\text{PO}_3^-$ residue is not known. This assumption is consistent with the anionic behavior of the complex. Complex B is stable at -20° in the dark in 10 mM KCl for at least 6 months.

The fact that this derivative is a low spin cobaltic complex has been confirmed by its nuclear magnetic resonance spectrum.

A low spin state of the cobalt ion would induce chemical shifts and little, if any, broadening of the resonance peaks. Such is indeed the case.

Labeling of Phosphorylase b—The labeling of phosphorylase *b* was done as follows: 10 mg per ml of enzyme were recrystallized in 20 mM AMP, 10 mM Mg^{2+} , and 50 mM Tris-acetate (pH 7.0) in the absence of thiol-containing agents. Under these conditions, all AMP sites of phosphorylase *b* are saturated. AMP-Co(III), Fraction B, was then added at about the desired stoichiometry (from 0 to 1 mole per mole of monomer) and the crystals were left for at least 2 days at 4° . After centrifugation, they were dissolved in the proper buffer and freed from AMP, magnesium, and eventually excess of AMP-Co(III) by passage through a Sephadex G-25 column. It was not possible to label phosphorylase at a stoichiometry higher than one, even with concentrations of AMP-Co(III) corresponding to a level higher than one per monomer of enzyme.

The amount of AMP-Co(III) bound to phosphorylase *b* was calculated from the ultraviolet spectrum by assuming additivity of absorbancies at 260 and 280 nm for the different chromophores (11). If x is the content in AMP-Co(III) per enzyme subunit, the ratio $p = A_{260}:A_{280}$ can be written as

$$p = \frac{0.70 + 0.22x}{1.32 + 0.14x}$$

The ratio p may therefore vary between 0.53 ($x = 0$) and 0.64 ($x = 1$). The assumption of additivity of absorbancies is confirmed by the fact that for a completely labeled enzyme, the ratio has been found equal to 0.64, whereas the cobalt content of the protein was found equal to one per subunit after displacement of the label by DTT and measuring of the corresponding absorbancy at 480 nm (Table I). Label can also be displaced by reduction with Fe^{2+} or sodium borohydride (after 5 min of incubation in a 0.1 mM solution of the reducing agent with 1 μg of phosphorylase *b* at room temperature).

In the crystals the labeled enzyme was stable for about 15 days at 4° . In solution, at pH 6.9 and 25° , 10% of the activity is destroyed after about 12 hours, which is not very different from a native enzyme, unprotected by DTT or 2-mercaptoethanol. The stability is higher at lower temperature (a drop in enzymatic activity of 10% is observed after 24 hours of storage at pH 7.2 and 5° or at higher pH). This allows us to do equilibrium dialysis measurements overnight at low temperature and activity tests at 28° . The direct labeling of phosphorylase *b* in solution seems very difficult since the presence of AMP-Co(III) (Fraction B) causes a rapid precipitation of the enzyme. Perhaps the flexibility of the protein in solution brings a reactive group in the vicinity of the AMP-Co(III) complex, whereas this event does not happen in the crystalline lattice.

Affinity Column Chromatography—A Sepharose-derived affinity column was synthesized according to the general procedure described by Cuatrecasas (12), by coupling hexamethylenediamine to activated Sepharose, having it react with *O*-bromoacetyl-*N*-hydroxysuccinimide and finally coupling $[^{14}\text{C}]\text{AMP}$ to it.

The local concentration of coupled nucleotide in the packed Sepharose was measured by counting an aliquot of the gel in Bray's solution. It was equal to 4 mM. Phosphorylase *b* readily adsorbed on the column and could be eluted by either 0.1 M AMP or 0.10 to 0.15 M glucose 6-phosphate in Tris-Cl, 0.1 M, at pHs ranging from 7.0 to 8.5.

Miscellaneous Methods—Equilibrium dialysis experiments were performed according to Myers and Schellman (7, 13). Each dialysis cell was formed of two chambers each having a volume

of about 300 μ l separated by a cellulose acetate membrane. The dialyses were performed at 5° for 16 hours in the presence of radioactive glucose-6-P or 5'-AMP and two 50- μ l aliquots withdrawn from each chamber were counted in 10 ml of Bray's scintillation dioxane solution in an Intertechnique SL-40 counter.

Enzymatic assays were performed in the standard assay me-

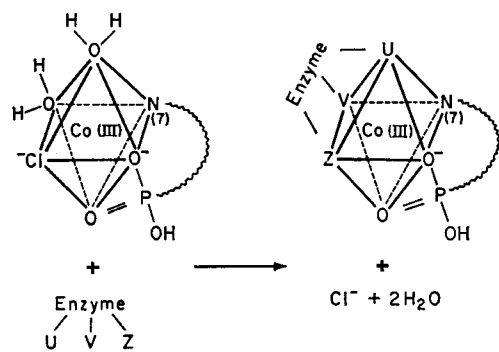


FIG. 3. Possible scheme of substitution of some positions of the AMP-Co(III). Coordination complex by electron-donor groups, U, V, Z of phosphorylase *b* (chelation by two oxygens of the phosphate group and by residue N (7) of the base in the AMP-Co(III) complex is only a working hypothesis).

TABLE II

Maximal enzymatic activity of phosphorylase *b* before and after removal of the label

The maximal activity in the absence of label V_{\max}^0 has been taken conventionally as equal to 1.0. The phosphorylase *b* concentration was 2.5 μ g per ml and the Fe^{2+} concentration was 0.1 mM. The incubation was performed in the standard assay medium devoid of 2-mercaptoethanol (glycogen, 2.5 g per liter; orthophosphate, 12 mM; Tris acetate, pH 7.0, 50 mM); 1 mM AMP was added after 5 min of preincubation. The $A_{340 \text{ nm}}$ increase is produced by the reduction of NADP (0.5 g per liter) upon action of glucose 6-phosphate dehydrogenase which oxidizes glucose-6-P produced by conversion of glucose-1-P by phosphoglucomutase.

Fraction of label per subunit	V_{\max}/V_{\max}^0	V_{\max}/V_{\max}^0 after removal of the label by Fe^{2+}
1	0	
0.50	0.46	0.94
0.40	0.61	
0.33	0.63	0.96
0.25	0.76	0.94

TABLE III

Influence of label on kinetic and dissociation constants of different effectors of phosphorylase *b*

The contribution of the hybrid species becomes more and more important as the concentration x of the label increases.

Fraction of label per subunit	$K_{1/2}(\text{glyc})^a$ (28°)	\bar{n}_{glyc} (28°)	$S_{5'-\text{AMP}}^c$ (5°)	$S_{\text{glucose-6-P}}^c$ (5°)	$K_{5'-\text{AMP}}^d$ (5°)	$K_{1(\text{glucose-6-P})}^e$ (5°)	$K_{2(\text{glucose-6-P})}^e$ (5°)
0	0.6	1.1	1.0	1.0	μM 7	μM 77	μM 32.5
0.25	0.9	1.7	0.75	0.75	8	50	32
0.50	1	1.8					
0.60				0.29		35	32

^a $K_{1/2}(\text{glyc})$, concentration of glycogen (in g per liter) corresponding to one-half the maximal activity V_{\max} ; 5'-AMP = 1 mM; orthophosphate = 36 mM.

^b \bar{n}_{glyc} , Hill coefficient of the curve $\log(V/V_{\max} - V) = f(\log(\text{glycogen}))$.

^c S , number of sites available per subunit for the noncovalent effector. This value is obtained from a Scatchard plot of the cor-

responding binding data. For a fully labeled enzyme, no binding of tritiated 5'-AMP is found at 5°.

^d $K_{5'-\text{AMP}}$, dissociation constant found by equilibrium dialysis at half-saturation for 5'-AMP (curves are slightly cooperative).

^e $K_{1(\text{glucose-6-P})}$ and $K_{2(\text{glucose-6-P})}$, first and second dissociation constants found for the binding of glucose 6-phosphate at 5°.

RESULTS

5'-AMP-Co(III) is Strongly Attached to Phosphorylase b—The centrifuged crystals of phosphorylase *b* which have been in contact with 5'-AMP-Co(III) (Fraction B) are of blue color. Moreover, after a passage through a G-25 column the color stays with the enzyme. This is a strong suggestion that at least one ligand of the octahedral field of the cobaltic ion now is a ligand donated by the protein (Fig. 3). Other cobaltic derivatives such as EDTA-Co(III) or aspartic acid-Co(III) do not color the crystals of phosphorylase *b*.

The enzymatic assays for maximum initial activity were performed on different preparations of labeled enzyme at different ratios of bound AMP-Co(III) per subunit. If x is the amount of bound label per subunit, the maximum activity is equal to $V_{\max}^0 \cdot x$ (V_{\max}^0 maximum activity in the absence of label) (Table II). This indicates that the number of inactivated subunits is equal to the amount of bound AMP-Co(III). When 1 mM EDTA-Co(III) or aspartic acid-Co(III) is added to the assay medium, there is no change in this maximum velocity. GTP-Co(III), electrolytically synthesized by a procedure similar to that used in the synthesis of AMP-Co(III) and purified by high voltage electrophoresis, was also tested; it had no influence on the enzyme activity. The effect of a similar ATP-Co(III) derivative on activity could not be tested since it precipitated the enzyme.

These results show the high specificity of the protein for the 5-AMP cobaltic derivative. Also, the number of inactivated subunits is equal to the amount of the bound label.

We have demonstrated (Table II) that the binding of the label did not irreversibly affect the structure of the phosphorylase, since reducing agents such as Fe^{2+} or sodium borohydride restore the maximum initial velocity V_{\max}^0 . This may also be shown by adding to the labeled enzyme 2-mercaptoethanol or DTT which, as we have seen, displace the cobaltic complexes from their original ligands.

Activator and Inhibitor Sites Are No Longer Accessible—Table III shows the results of equilibrium dialysis performed at 5° with enzymes labeled with nonradioactive AMP-Co(III) at different stoichiometries (x) per monomer in the presence of

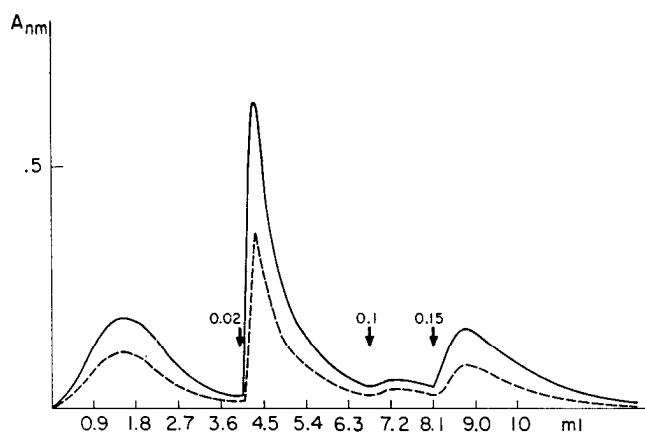


FIG. 4. Elution profile of half-labeled phosphorylase *b* with 5'-AMP-Co(III) on a Sepharose-5'-AMP affinity column. The elution is performed in Tris-HCl (pH 8.2, 0.1 M). The total amount of phosphorylase *b* is 1 mg. The first eluted peak is made of phosphorylase *b* labeled with one 5'-AMP-Co(III) on each subunit. When 0.02 M of the allosteric inhibitor glucose-6-P is added, a half-labeled enzyme is eluted. The relative intensities of the peaks are: 1.1; 1.77, 1.0. The initial amount of protein has been recovered at the precision of the experiment. The solid line follows the 260-nm absorbance, while the dotted line follows the 280-nm absorbance.

[^3H]5'-AMP or [^{14}C]glucose-6-P in Tris-acetate (pH 7.0, 50 mM). Scatchard plots extrapolate in both cases to values equal to $1 - x$ times the concentration of subunits. Thus, as in the assay for maximum initial velocity, the number of subunits unable to bind [^3H]5'-AMP or [^{14}C]glucose-6-P is exactly equal to the amount of bound AMP-Co(III).

Separation of Three Classes of Labeled Phosphorylase May Be Achieved on a 5'-AMP-Sepharose Column—When phosphorylase *b* is partially labeled, three types of dimer may coexist: nonlabeled (0:0), half-labeled (0:1), and fully labeled (1:1) molecules. The measured constants for the hybrid molecules, therefore, contain a contribution from the nonlabeled ones. We tried to separate these classes in order to evaluate the true concentration of hybrid molecules present in the mixture.

In order to avoid subunit exchange on the column, we ran the experiment at pH 8.2, since monomerization of phosphorylase *b* is relatively slow at alkaline pH (14). Enzyme labeled with less than one AMP-Co(III) per monomer was layered on top of the column in Tris-chloride (0.1 M, pH 8.2) and eluted with steps of increasing concentrations of glucose-6-P.

Fig. 4 shows that three peaks were obtained. The first one had no activity and was (1:1) labeled (it recovered its activity after incubation with 10 mM 2-mercaptoethanol); the second peak was eluted by 0.02 M glucose-6-P and was a hybrid (0:1) molecule as seen from its $A_{260}:A_{280}$ ratio; the third peak was eluted in the presence of 0.10 to 0.15 M glucose-6-P (depending on the experiment) and was unlabeled phosphorylase *b*.

In an experiment where the initial mixture was 0.25 labeled, the relative proportion of the peaks was 1, 5.5 and 9.5; in the case of a 0.50 labeling, the relative proportion of the peaks was 1, 1.65, and 0.93. This shows that the distribution of initial labels is random, within the precision of the method. If the same experiment is made at pH 7.0 in Tris-acetate (0.05 M), a gradient of glucose-6-P elutes the labeled phosphorylase *b* in a single broad peak which is more labeled in the first fraction than the last ones; this indicates that significant subunit exchange takes place under these conditions. For a 1 mg per ml solution the extent of hybrid formation is not significant after 100 min

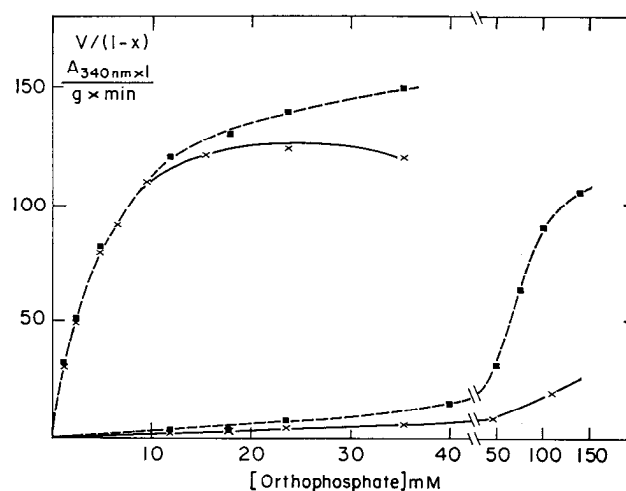


FIG. 5. Dependence of the initial velocity of the reaction as a function of orthophosphate concentration in the absence (lower curves) or presence (upper curves) of 1 mM AMP. ■, the native enzyme; ×, phosphorylase *b* labeled at a ratio of 0.84 AMP-Co(III) per subunit. The ordinates are normalized in order to take into account the percentage of catalytically active subunits. The glycogen concentration is 2.5 g per liter. The crystalline suspension of auxiliary enzymes brings a concentration of 16 mM ammonium sulfate; this is also the case in Fig. 6 and Table III.

at 22° at pH 8.2, while, at the same temperature and at pH 7.0, this formation is about complete after the same period of time.²

Effect of Label Extends to Vicinal Unlabeled Subunit—We wished to compare the kinetic and binding properties of an hybrid phosphorylase *b* dimer with those of the native enzyme. Since the distribution of the labels appears to occur at random, we have labeled with AMP-Co(III) an enzyme population slightly under the theoretical stoichiometry. For instance, when the fraction of labeling x is 0.84, one expects to get 70% of the doubly labeled inactive dimer, 2.5% of the native enzyme and 27% of hybrid molecules; accordingly, under such conditions there is a 10-fold excess of the interesting class over the unlabeled one. The kinetic response of this mixture will thus correspond to the behavior of the isolated purified hybrid, with an error of less than 20%. One should note that isolation of purified hybrids would not have improved this situation since the labeled subunits redistribute at random at pH 7.0 as we observed in the preceding section.

The initial velocity of glycogen phosphorolysis as a function of orthophosphate concentration was studied under two different conditions: at saturation in AMP (1 mM) and in the absence of AMP. Fig. 5 shows that, at saturation in AMP, the curve is hyperbolic (it also appears an inhibition phenomenon by excess orthophosphate) and the corresponding Michaelis constant is close to that of the native enzyme (5 mM in both cases). However, in the absence of AMP, the hybrid molecule responds much more poorly to orthophosphate: the initial velocity is very small and proportional to the orthophosphate concentration up to at least 0.1 M; moreover, one observes that the kinetic response of the dimer to the phosphate parallels the response to very low concentrations of orthophosphate of the native enzyme.

² We have used glucose-6-P instead of AMP because of its low ultraviolet absorption and because it is known to prevent AMP binding by displacement of the allosteric equilibrium. One can try to extend this method to the purification of allosteric enzymes, since it selects in a single step proteins which have two distinct stereospecific sites.

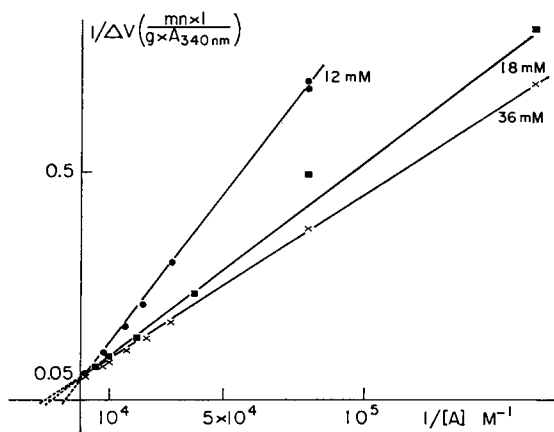


FIG. 6. Kinetic behavior of phosphorylase *b* labeled at a ratio of 0.84 AMP-Co(III) per subunit. The glycogen concentration is 2.5 g per liter. Other conditions are as indicated under Table II. V is the difference between the initial velocities taken in the presence of a concentration (A) of AMP, and the initial velocity in the absence of activator. Each line corresponds to the indicated orthophosphate concentration. One observes that the experimental points fall on *straight lines* at the precision of the experiment; this shows that the influence of AMP on the activity is noncooperative in the case of hybrid phosphorylase *b* molecules.

TABLE IV

Concentration of AMP required for half-activation of phosphorylase *b* for increasing degrees of labeling x with AMP-Co(III)

The velocity assays are performed in a medium containing 2.5 g per liter of glycogen. The response is cooperative for $x = 0$ or $x = 0.25$ and hyperbolic for $x = 0.84$.

Orthophosphate	Control $x = 0$	$x = 0.25$	$x = 0.84$
mM	μM	μM	μM
12	12	42	103
18	8	19	61
36	5	13	52

Fig. 6 shows that, at a given orthophosphate concentration, the response of the initial velocity to AMP is hyperbolic; this is not the case of the unlabeled enzyme where this response is sigmoidal (at least for the lower orthophosphate concentrations). Table IV shows, furthermore, that the concentration required for half-activation is much larger for the hybrid molecules than for the unlabeled enzyme. The reciprocal effect between AMP and orthophosphate which has been observed for the native enzyme (7, 15), is maintained in the hybrid dimer since AMP decreases the Michaelis constant for orthophosphate while increased concentrations of substrate decreases the concentration of the nucleotide required for half-activation.

The kinetic response to glycogen has been studied at saturating concentrations of AMP and orthophosphate. Fig. 7 shows that, for a ratio of AMP-Co(III) to phosphorylase *b* subunits equal to 0.5, unlike in the control, the binding of glycogen is no longer hyperbolic but sigmoidal. It is characterized by a maximum Hill coefficient of the order of 1.8, around the half-saturation value in glycogen which corresponds to a substrate concentration of 1 g per liter. At very high glycogen concentrations, the Hill plot is expected to reach a final asymptotic value of Slope 1; the position of this asymptote gives in theory the Michaelis constant of the last molecule of glycogen bound on the dimer. An accurate value of this constant cannot be derived from the present data since the curvature of the Hill plot is barely apparent on Fig. 7, in a range where the experimental error is large. This curve

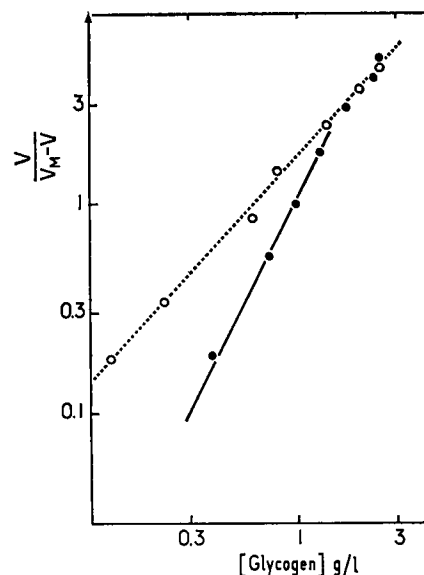


FIG. 7. Hill plot for the dependence of the initial velocity of the phosphorolysis reaction with respect to glycogen. Phosphorylase *b* is labeled at a ratio of 1 AMP-Co(III) per two subunits. The orthophosphate concentration was 36 mM and that of AMP 1 mM. The maximal Hill coefficient taken on the first four points is 1.83 ± 0.08 and the average slope for the seven points is equal to 1.70 ± 0.05 . The solid line is for the labeled enzyme, whereas the dotted line (O---O) corresponds to the unlabeled species.

either crosses the straight line which corresponds to the control experiment ($x = 0$) or at worst reaches it asymptotically. It appears therefore that the final Michaelis constant is smaller or equal to that of the control (0.6 g per 1 liter).

Finally, Table III illustrates another characteristic feature of the quaternary effect of the label: at a moderately high fraction of labeling ($x = 0.6$) the binding of radioactive glucose 6-phosphate becomes noncooperative, the association constant being equal to the second binding constant of this effector for unlabeled phosphorylase *b* (16). It appears therefore that labeled hybrids bind more strongly the inhibitor glucose 6-phosphate and require higher concentrations of the activator to be catalytically fully active.

DISCUSSION

We have shown that it is possible to synthesize a non-exchangeable complex between AMP and cobaltic ion by electrolytic oxidation of the labile cobaltous complex. This strongly colored derivative is stable in solution. The same oxidation procedure has been extended to similar compounds such as ATP-Co(III), GTP-Co(III), dAMP-Co(III), or 3'-5'-AMP-Co(III).

The 5'-AMP-Co(III) complex can be inserted in phosphorylase *b* and has the double advantage of being strongly bound to the enzyme and yet of being displaced at will by thiol compounds. The stoichiometry of the labeling does not exceed one per monomer in the case of phosphorylase *b*. Other cobaltic derivatives such as EDTA-Co(III), aspartic acid Co(III), or GTP-Co(III) have no influence on the enzyme activity and do not label the crystals, indicating that the labeling is highly specific and strongly suggesting that the label is bound at the AMP site. The fact that the activity is restored after removal of the label shows that the structure of the protein has not been irreversibly altered. The same features—high stereospecificity and restoration of the activity after thiol attack—have been tested with other Co(III) label systems and will be the subject of further publications.

Once the cobaltic label has been attached to the enzyme, a new pattern of interaction appears. The labeling destroys the catalytic activity of the labeled subunit and prevents the association of AMP and glucose-6-P on it. In addition the response to glycogen is highly cooperative at saturation in AMP and orthophosphate. This observation indicates a modification in the quaternary structure of the labeled enzyme. At high glycogen concentrations, the final Michaelis constant for glycogen is smaller or equal to that of the control.

Therefore, the binding site for glycogen in the labeled subunit is at least partially preserved and glycogen binding on the labeled subunit, where it is not degraded, is required for a proper association of the polysaccharide on the other subunit.

Equilibrium dialysis experiments performed with glucose 6-phosphate indicate a second change in quaternary structure, since the presence of the 5'-AMP-Co(III) label on one subunit favors the binding of glucose-6-P on the other one. More precisely, only unlabeled subunits can bind 5'-AMP or glucose-6-P, and the single dissociation constant which characterizes glucose-6-P binding is found equal to the second dissociation constant of the native enzyme for this effector. This observation is consistent with an effect of the label at the inhibitor site of the unlabeled subunit mediated by a quaternary structural change and similar to the one triggered by the binding of the first molecule of glucose 6-phosphate on the native dimer (16).

In the hybrid phosphorylase *b* molecule all of the sites are preserved in the unlabeled subunit. The initial velocity measurements performed at saturation in AMP and in glycogen indicate that the turnover number and the Michaelis constant for orthophosphate of the unlabeled subunit of the hybrid dimer are close to those of the unlabeled enzyme. However, higher concentrations of AMP are required to reach half-activation and, in the absence of AMP, the degree of phosphorylation is very poor, due to the very small affinity of orthophosphate. As the orthophosphate concentration is increased the requirement for AMP is lowered. This demonstrates that the positive interaction between orthophosphate and AMP which is present in the native enzyme is preserved in the unlabeled subunit of the hybrid dimer. Accordingly, besides quaternary changes we have demonstrated a strong linkage between the catalytic and activator sites in each subunit of the hybrid. The catalytic activity and the activator sites are destroyed in the labeled subunit while a positive linkage between orthophosphate and AMP is maintained in the unlabeled one. It is therefore quite likely, as suggested by other authors, that catalytic and regulatory sites are very close in the tertiary structure and that a slight modification of any of them has a strong and direct influence on all the others (17).

Other transition metal-nucleotides derivatives have been proposed as analog of substrates; however they seem to show no interaction with the proteins tested (18, 19). 5'-AMP analogs have also been recently proposed for the labeling of the activator sites of phosphorylase *b*. Minor changes in the design of the label can lead either to activation or to inhibition. Thus, Hulla and Fasold (20) have synthesized 6-(purine-5'-ribonucleotide)-5-(2-nitrobenzoic acid) thioether which binds covalently to glycogen phosphorylase after elimination of 2-nitro-4-mercapto-benzoic acid. Activation shows a stoichiometric relation to the amount of incorporated nucleotide. Graves³ has recently reported that adenine substituted at Position 8 by an —S-

CH₂-Ar-NH-C-Ar-SO₂F side chain can, after reaction with en-



zyme, lead either to activation or to inhibition depending on the position of the sulfonyl fluoride residue on the second aromatic ring. In collaboration with Cooperman we have also used *O*'-(ethyl 2-diazomalonyl)5'-AMP, a photoaffinity label which abolishes enzymatic activity (21). Characterization of the peptides attached to some of these ligands after tryptic analyses is now underway. More generally, we have shown that nucleotides bound to Co(III) can afford new ways of labeling stereospecific sites. These labels are conditional since they can be removed at will by thiol-containing molecules but are otherwise stable. This kind of conditional label can probably be used to isolate a large spectrum of enzymes requiring both Mg²⁺ and a given substrate at vicinal sites. Moreover, as in the case of nucleic acids (9), it is likely that such a label can provide a means of investigating the local features of the binding sites. Coupled with affinity chromatography, this method allows the isolation of incompletely labeled oligomeric proteins and offers a new way of analyzing interaction patterns between different stereospecific sites in proteins.

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³ D. J. Graves, Report at the Second Symposium on Metabolic Interconversion of Enzymes, Rottach Egern, Bavaria (1971).