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Dinitrophenylation of Glycogen Phosphorylase. I. Preparation and Properties of Active Dinitrophenyl Derivatives*

George Philip and Donald J. Graves†

ABSTRACT: Dinitrophenylation of phosphorylase *b* with an eightfold molar excess of 2,4-dinitrofluorobenzene (DNFB) results in inactivation of the enzyme, and subsequent analyses of the protein showed modification of four to five groups. ε-Amino groups of lysine and SH groups of cysteine were modified. In the presence of either α-D-glucose 1-phosphate (glucose-1-P) or adenosine 5′-monophosphate (AMP), inactivation is retarded and in the presence of both, 75% of the activity could be retained with modification of 3–3.5 groups. When glucose-1-P or AMP was present during dinitrophenylation, their respective binding sites were preserved as indicated by kinetic studies DNP-phosphorylase *b*, prepared in the presence of glucose-1-P or AMP or both, could be converted into crystalline DNP-phosphorylase *a* derivatives. DNP-phosphorylase *a* prepared from phosphorylase *b* dinitrophenylated in the presence of glucose-1-P and AMP was found to be electrophoretically homogeneous and sedimented essentially as a single component in the ultracentrifuge. No change in K_M for glucose-1-P and AMP was observed with this derivative in comparison with the unmodified enzyme but a clear difference existed in the K_M for glycogen.

In order to delineate the importance of specific amino acid residues in phosphorylase structure and activity, the susceptibility of this enzyme to a chemical modifying agent, DNFB,[1] has been tested. Although reactions of phosphorylase with chemical agents have not been used extensively, Madsen and Cori (1956) showed that modification of nine cysteinyl residues of phosphorylase *b* dimer resulted in formation of an inactive monomer. More recently, studies of phosphorylase with other sulfhydryl reagents (Kudo and Shukuya, 1964; Damjanovich and Kleppe, 1966; Damjanovich *et al.*, 1967) further defined the role of cysteinyl residues in catalysis. The involvement of lysyl residues in structure and activity has been demonstrated by Huang and Madsen (1966); their studies with cyanate showed that carbamylation of 23 ε-amino groups of lysine in phosphorylase *b* resulted in enzyme inactivation and dissociation.

Dinitrophenylation of phosphorylase was chosen since ribonuclease (Hirs, 1962), aldolase and transaldolase (Rowley *et al.*, 1964; Kowal *et al.*, 1965), and fructose 1,6,-diphosphatase (Pontremoli *et al.*, 1965), all enzymes like phosphorylase in that their substrates are anionic, could either be inactivated or converted into a different catalytic form by dinitrophenylation of a small number of amino acid residues.

The work reported herein, shows that the lysyl residues can be preferentially modified under specific conditions and that inactivation can be accomplished by modification of a small number of groups. Under somewhat different experimental conditions, inactivation results largely from modification of sulfhydryl groups (Gold, 1968).

Experimental Section

Materials. Phosphorylase *b* from rabbit skeletal muscle was prepared according to the procedure of Fischer and Krebs (1958) but mercaptoethanol was substituted wherever cysteine was required. Three-times-recrystallized phosphorylase *b* was used in this work. Phosphorylase *b* was converted into phosphorylase *a* by using phosphorylase *b* kinase as described by Fischer and Krebs (1962). Enzyme used for dinitrophenylation was first dialyzed for 2 days against 0.025 M Tris-HCl buffer (pH 7.6) at 3–4° to remove mercaptoethanol. The specific activities of a typical preparation of this enzyme were 1700 and 1250 Cori units/mg, respectively, when measured according to the procedure of Illingworth and Cori (1953) in 0.015 M cysteine–0.02 M glycerophosphate (pH 6.8) and in 0.0125 M

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: DNFB, 2,4-dinitrofluorobenzene; PMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Tris-HCl (pH 6.8). The dialyzed enzyme showed only 12 titratable SH groups in 8 M urea with DTNB (Ellman, 1959) based on a molecular weight of 190,000 (Fischer *et al.*, 1967) in contrast to 16 titratable groups for a control sample of phosphorylase *b* (native enzyme that was treated with dithiothreitol followed by gel filtration on a column of Sephadex G-15).

AMP was purchased from Pabst Laboratories, and Tris from Fisher Scientific Co. Cysteine-HCl, sodium glycerophosphate, potassium glucose 1-phosphate, shellfish glycogen, PMB (sodium salt), DNFB, and ε-DNP-lysine were products of Sigma Chemical Co. *S*-DNP-cysteine was purchased from Nutritional Biochem. Corp. DNFB, uniformly labeled with ^{14}C, was obtained from Nuclear-Chicago Corp. Frozen rabbit muscle was obtained from Pel-Freez Biologicals, Inc. The glycogen and enzyme solutions were treated with Norit A to remove contaminating AMP.

Methods. The reaction of phosphorylase *b* with DNFB was initiated by adding 0.02 ml of a 0.05 M solution of DNFB in ethanol to 5 ml of phosphorylase (5 mg/ml). At various intervals, aliquots were removed and diluted in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8) to stop inactivation and then incubated for 0.5–1 hr prior to measurement of enzymic activity.

For determination of initial velocities, enzyme solutions were preincubated at room temperature for 1 hr in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8) prior to rate measurements. Reactions with phosphorylases were run at 30°, with substrate containing 1% glycogen, glucose-1-P, and AMP as indicated. Aliquots were removed and analyzed for inorganic phosphate according to Fiske and Subbarow (1925). Sampling times were adjusted to ensure linear product *vs.* time curves. Specific activities are expressed as micromoles of phosphate released per minute per milligram of enzyme. The concentrations of native enzyme were determined spectrophotometrically using an absorbance index of 11.7 for a 1% solution of protein (Velick and Wicks, 1951) and also colorimetrically using Biuret reagent (method A) calibrated against serum albumin (Chaykin, 1966). Protein concentrations obtained by these two methods did not vary more than 3–4%. Since DNFB did not interfere with the Biuret method, this procedure was used for the determination of the concentration of DNP-phosphorylases. Sulfhydryl groups were estimated according to Ellman (1959) in phosphate buffer (pH 8.0).

Ultracentrifuge runs were performed on a Spinco Model E at a rotor speed of 59,780 rpm and a temperature of 25°. Sedimentation coefficients were determined with the aid of a Nikon Model 6C microcomparator and were corrected for viscosity and density of the buffer to water at 20°.

Radioactivities were measured in planchets using a gas-flow counter Model C-110B of Nuclear-Chicago Corp. For determination of the number of types of groups modified, phosphorylase *b* was allowed to react with [^{14}C]DNFB and the reaction was stopped by addition of neutral cysteine. The resulting solution was passed through Sephadex G-25 and the protein fractions were collected. After determining the protein concentration, a small portion was dried and counted. The number of the DNP groups incorporated into the protein was calculated from the specific radioactivities of the DNP protein and the DNFB solution. The concentration of the DNFB solution was determined according to Hill and Davis (1967). A known volume (0.5 ml) of DNFB solution in Tris-HCl (pH 7.6) was shaken with 20 ml of a 1% solution of benzylamine in toluene for 30 min at room temperature. The toluene layer was filtered through glass wool, and the absorbance was measured at 343.5 mμ against a blank treated similarly. The concentration of DNFB was calculated using an extinction coefficient of 17,300. The number of DNP groups incorporated into phosphorylase *b* was also determined by estimating unreacted DNFB before and after reaction with protein, using the above method.

For the determination of groups modified, the DNP enzyme solution was made 10% in trichloroacetic acid in the cold. The precipitated protein was collected by centrifugation and washed several times with 0.5 M HCl. The DNP enzyme was hydrolyzed with constant-boiling HCl in a sealed and evacuated tube for 24 hr at 100–110°. The hydrolysate was thoroughly extracted with ether and the ether layer which was devoid of any DNP-amino acid was discarded. The aqueous layer was flash evaporated at 60°, and the residue was dissolved in water and again dried. This was repeated at least six times. The final residue was analyzed by column chromatography on a 1 × 6 column of Hyflo Super-Cel according to Matheson (1966), paper chromatography on Whatman No. 3MM paper using *t*-amyl alcohol saturated with 0.05 M phthalate buffer (pH 6.0) as solvent (Porter, 1957), and also by high-voltage paper electrophoresis at 3000 V, in 1.5 M formic acid (pH 1.9) on 56 × 15 cm Whatman No. 3MM filter paper (Kowal *et al.*, 1965).

Disc gel electrophoresis was carried out according to Ornstein and Davis (1961) using 4×10^{-2} M asparagine adjusted to pH 7.3 with 2 M Tris and 0.15 M Tris-HCl (pH 7.9) as upper and lower buffers, respectively. A 7.5% gel was used in columns of 5 mm × 4 cm. The entire apparatus was refrigerated at 3° and a current flow of 3 mA/tube was maintained for 5 hr. Protein was detected by staining with Amido Black. Activity was detected by formation of calcium phosphate in the gel (Allen and Hyneik, 1963).

Results

Dinitrophenylation of Phosphorylase b. Incubation of phosphorylase *b* at pH 7.6 with a eightfold molar excess of DNFB results in the loss of enzyme activity (Figure 1). The rate of this inactivation is effectively slowed when either glucose-1-P (0.016 M) or AMP (0.002 M) is present in the reaction mixture. A 50% inactivation required only 15 min in the absence of substrate, or activator but more than 200 min in the presence of either glucose-1-P or AMP. Figure 1 also shows the enormous protective effect against inactivation when both glucose-1-P and AMP were present in the reaction mixture. In this case, DNFB brought about

25% inactivation in the first 50 min (at a still slower rate than when either the substrate or activator was present) but was without effect on continued incubation.

Investigation of the number and types of amino acid residues modified was undertaken in order to be able to define the DNFB reaction more specifically. The number of residues modified was calculated from the specific radioactivities of the protein and the DNFB solution used and also from the number of moles of unreacted DNFB before and after the reaction. The results are illustrated in Table I. In the absence of substrate and activator, modification of four to five residues resulted in 85–95% of inactivation. The variation observed in different experiments is due in part to the difficulty of stopping the reactions always at the same extent of inactivation. Moreover, slight variations in the results were observed between different enzyme preparations which no doubt is a result of varying degrees of oxidation of sulfhydryl groups (see Materials). The table also shows the agreement between the results obtained with radioactive DNFB and the benzylamine method. When dinitrophenylation was carried out in the presence of AMP and glucose-1-P, modification of 3–3.5 residues resulted in only 25–30% inactivation. Less groups are modified in the presence of AMP and glucose-1-P even if the reaction is carried out 20 min longer than the reaction in their absence; these results clearly show that glucose-1-P and AMP block reaction of certain groups which would have otherwise readily reacted.

In order to identify the amino acid residues modified,

TABLE I: Number of DNP Groups Incorporated per Mole of Protein.[a]

	Phosphorylase *b* Dinitrophenylated in the Presence of		
Expt	No Addn[b]	AMP and Glucose-1-P[c]	DNP-phosphorylase *a*[d]
1	4.2	3.5	4.5
2	4.7, 4.9[e]	3.1	4.3
3	5.1	3.5	4.7
4	5.4[e]		

[a] The number of DNP groups per mole of protein was calculated from specific radioactivities of DNFB solution (except those superscribed with *e*) and the DNP protein using molecular weights of 190,000 for phosphorylase *b* and 380,000 for phosphorylase *a* (Fischer *et al.*, 1967). [b] The reaction was stopped at 120 min by addition of neutral cysteine. The samples used for analysis were 85–95% inactivated. [c] Reactions were stopped at 25–30% inactivation (120–140 min). [d] DNP-phosphorylase *a* was prepared from phosphorylase *b* dinitrophenylated in the presence of AMP and glucose-1-P. [e] Results obtained with benzylamine method.

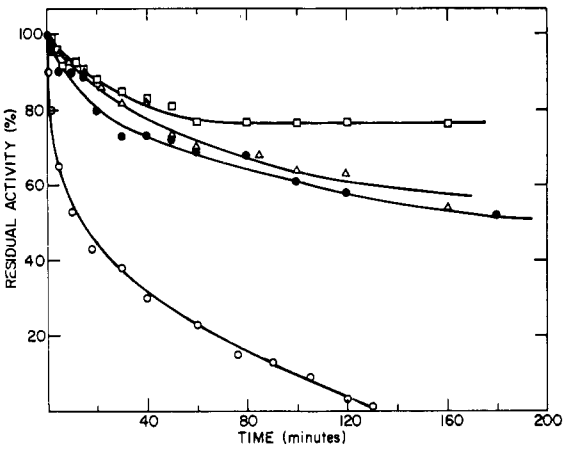

FIGURE 1: Rate of inactivation of phosphorylase *b* with DNFB. Reaction mixtures contained phosphorylase *b* (2.6×10^{-5} M) and 2×10^{-4} M DNFB in 0.025 M Tris-HCl buffer (pH 7.6). No additions (○); with 2×10^{-3} M AMP (△); with 0.016 M glucose-1-P (●); with 0.016 M glucose-1-P and 2×10^{-3} M AMP (□). Aliquots were removed at various intervals and tested for enzymic activity.

DNP derivatives of phosphorylase *b* dinitrophenylated with [^{14}C]DNFB in the presence and absence of AMP and glucose-1-P were hydrolyzed and analyzed. The hydrolysates were extracted thoroughly with ether; the ether extracts which were devoid of any DNP-amino acid were discarded. Analyses of the aqueous layers on Hyflo Super-Cel according to Matheson (1966) and by paper chromatography (Porter, 1957) showed essentially one yellow component only, the rate of migration of which was identical with that of ε-DNP-lysine. However, a small streak was occasionally detected on the paper chromatogram. Moreover, irregularities were observed in migration of spots on chromatography and electrophoresis of the residue, probably due to the presence of salts, amino acids, and their decomposition products. This was avoided by purifying the hydrolysate by paper chromatography. The radioactive area was cut out, eluted, and concentrated. When this purified material was used for high-voltage electrophoresis, two spots, one corresponding to ε-DNP-lysine and the other corresponding to *S*-DNP-cysteine, were detected.[2]

The number of lysyl and cysteinyl residues modified is listed in Table II. In the absence of AMP and glucose-1-P inactivation was caused by modification of an average of approximately 3 lysyl and 1.5 cysteinyl residues. Estimation of sulfhydryl groups of phosphorylase *b* in 8 M urea with DTNB before and after the reaction with DNFB showed 12.0 and 10.3 titratable groups, respectively, thus substantiating the above results. In the presence of substrate and activator, less than one (0.8) cysteinyl residue was modified when 2.5 lysyl residues were allowed to react with DNFB.

Since Shaltiel (1967) recently showed that thiolysis of *S*-DNP-cysteine occurs at pH 8 and because cysteine

[2] The use of [^{14}C]DNFB in this work enabled us to identify *S*-DNP-cysteine as a minor component which was not found in the earlier study (Philip and Graves, 1967).

TABLE II: Relative Modification of Lysyl and Cysteinyl Residues.[a]

Expt	Phosphorylase *b* Dinitrophenylated in the Presence of: No Additions — Lysyl Residue	No Additions — Cysteinyl Residue	AMP and Glucose-1-P — Lysyl Residue	AMP and Glucose-1-P — Cysteinyl Residue	DNP-phosphorylase *a* — Lysyl Residue	DNP-phosphorylase *a* — Cysteinyl Residue
1	2.8	1.4	2.8	0.7	3.6	0.9
2	3.0	1.7	2.3	0.8	3.4	0.9
3	3.8	1.3	2.5	1.0	3.8	0.9
Av	3.2	1.5	2.5	0.8	3.6	0.9

[a] After high-voltage paper electrophoresis, the spots corresponding to DNP-lysine and DNP-cysteine were cut out, eluted, and counted. From the ratio of radioactivities, the number of lysyl and cysteinyl residues modified were calculated using the results of Table I.

was used to stop the reaction, experiments were carried out to determine whether there was any thiolysis of DNP-phosphorylase under our experimental conditions. Phosphorylase *b* was dinitrophenylated as usual and half of the sample was treated with 0.01 M cysteine at pH 7.0 and 30° for 2 hr. This solution was then passed through a Sephadex column of G-15 and the protein fractions were collected. The extinction coefficients at 335 and 360 mμ were determined and compared with that of the sample not treated with cysteine but subjected to gel filtration. The extinction coefficients were found to be the same for both, showing that no detectable thiolysis occurred under these conditions. Moreover, no thiolysis was detected even in a sample of DNP enzyme treated with cysteine or mercaptoethanol at pH 8.0.

Since lysyl and cysteinyl residues were found to react with DNFB, further studies were carried out to determine the importance of each of these for activity. This was done by taking advantage of the fact that the PMB reaction is completely reversed by cysteine (Madsen and Cori, 1956) while the dinitrophenylation reaction is not. Equal volumes (5 ml of phosphorylase *b* (5 mg/ml) in 0.025 M Tris-HCl (pH 7.6) were taken in separate tubes and one of them was treated with a 12-fold molar excess of PMB in the same buffer, while buffer alone was added into the other. When the enzyme in the first tube was completely inactivated as determined by assaying in Tris-HCl buffer (pH 6.8), 20 μl of a 0.05 M DNFB solution was added into each tube and aliquots were withdrawn at intervals and treated with cystine–glycerophosphate buffer (pH 6.8) for 40–45 min, before assaying for catalytic activity. Blanks were run to check the stability of the enzyme and the reversibility of the PMB reaction. The results given in Table III show that phosphorylase *b* which was allowed to react with PMB, prior to addition of DNFB, showed the same rate of inactivation as that for the control (column I). The PMB inactivation was almost completely reversed on treatment with cysteine and the various forms of the enzyme were stable during the entire period of experiment. These results indicate that inactivation of phosphorylase *b* was likely due to dinitrophenylation of lysyl residues and that the cysteinyl residues, which reacted with DNFB, do not appear important for activity.

TABLE III: Rate of Inactivation of Phosphorylase *b* and PMB-phosphorylase *b* with DNFB.

Time (min)	Specific Activities[a]: Phosphorylase *b* Treated with DNFB	PMB-phosphorylase[b] Treated with DNFB
2	22.0	23.0
10	15.0	16.3
30	13.6	13.6
65	12.5	12.5
90	9.0	9.0

[a] Aliquots were preincubated with 0.03 M cysteine–0.04 M glycerophosphate (pH 6.8) for 30 min (30°) and assayed for enzyme activity (Illingworth and Cori, 1953). Specific activities are expressed in micromoles of inorganic phosphate liberated per minute per milligram of enzyme. [b] Phosphorylase *b* inactivated with PMB.

Kinetic Properties of DNP-phosphorylase b. The Michaelis constants for different DNP derivatives of phosphorylase *b* were investigated in order to determine the effect of chemical modification on the binding properties of the enzyme. Phosphorylase *b* inactivated 85%, as measured at fixed substrate concentrations (Illingworth and Cori, 1953), showed upon kinetic analysis a fourfold decrease in V_M and a higher K_M (1.8×10^{-2} M) as compared with the native enzyme (7.5×10^{-3} M) for glucose-1-P. Since a change in K_M could indicate alterations in the binding site, studies were undertaken to determine whether substrate and activator could maintain the characteristics of their

TABLE IV: Michaelis Constants for Unmodified and Modified Phosphorylase *b* Derivatives.

	Unmodified Phosphorylase *b* (M)	Phosphorylase *b*, Dinitrophenylated in the Presence of		
		Glucose-1-P[a] (M)	AMP[a] (M)	AMP and Glucose-1-P[c] (M)
K_M[b] for glucose-1-P	6 2–7 × 10^{-3}	8 × 10^{-3}	2.6 × 10^{-2}	6.3 × 10^{-3}
K_M[b] for AMP[d]	4–5 × 10^{-5}	1.9 × 10^{-4}	5 × 10^{-5}	5.5 × 10^{-5}

[a] Enzyme inactivation was stopped at 50% by dilution in glycerophosphate–cysteine buffer. [b] K_M's for glucose-1-P were determined with assay mixtures which contained 1% glycogen, 10^{-3} M AMP, glucose-1-P (0.008–0.03 M) in 0.015 M cysteine–0.02 M glycerophosphate buffer (pH 6.8). K_M for AMP with 0.016 M glucose-1-P, AMP (5 × 10^{-5}–10^{-3} M), and glycogen and buffer as above. [c] Reaction was stopped at 25% inactivation. [d] Although double-reciprocal plots can be best approximated by straight lines at these concentrations, all the derivatives showed nonlinear double-reciprocal plots at lower AMP concentrations as reported for native phosphorylase *b* (Sealock and Graves, 1967).

respective binding sites. The results are presented in Table IV. Enzyme partially inactivated in the presence of glucose-1-P showed a higher K_M for AMP (1.9 × 10^{-4} M) as compared with unmodified phosphorylase *b* (5 × 10^{-5} M). There was no appreciable change in K_M for glucose-1-P. Similar studies with enzyme modified in the presence of AMP showed that K_M for glucose-1-P was increased approximately fourfold as compared with the control, whereas the K_M for AMP, in this case, remained unchanged. Thus it appears that the substrate and activator when present in the reaction mixture during dinitrophenylation preserve their respective binding sites. Further support to this view is provided by the fact that no appreciably change in K_M's for glucose-1-P and AMP was observed when both substrate and activator were included in the reaction mixture.

Preparation and Properties of DNP-phosphorylase a. Since the derivative obtained in the presence of glucose-1-P and AMP was found to be electrophoretically heterogeneous (Figure 2) and could not be crystallized with Mg^{2+} and AMP (Fischer and Krebs, 1958), the purification of DNP-phosphorylase was attempted by conversion of this derivative into phosphorylase *a*.

Dinitrophenylation was carried out with a solution of 20 mg/ml of phosphorylase *b* in 0.025 M Tris-HCl (pH 7.6) containing 2 × 10^{-3} M AMP and 0.032 M glucose-1-P by addition of a eightfold molar excess of DNFB at 30°. When approximately 3 moles of DNP groups was incorporated/mole of enzyme, the reaction was stopped by addition of neutral cysteine. The DNP-phosphorylase *b* obtained had 70–75% of its original activity. The solution was dialyzed against 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8) followed by treatment with Norit A to remove the remaining AMP. The resulting DNP-phosphorylase *b* was allowed to react with phosphorylase *b* kinase and ATP for conversion into DNP-phosphorylase *a* according to Fischer and Krebs (1962). After the reaction was completed, the reaction mixture was made 50% with respect to ammonium sulfate and the precipitated protein was collected by centrifugation. It was dissolved in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8) and dialyzed against the same buffer overnight. The DNP-phosphorylase *a* was obtained in a yellow crystalline form and was recrystallized several times from glycerophosphate–cysteine buffer. A photograph of the crystals is shown in Figure 3. Analyses showed that 4.3–4.7 DNP groups were present/mole of phosphorylase *a* (Table I) and that the ratio of lysyl to cysteinyl residues modified was 4:1 (Table II). Since in the conversion of phosphorylase *b* into *a* there is a doubling

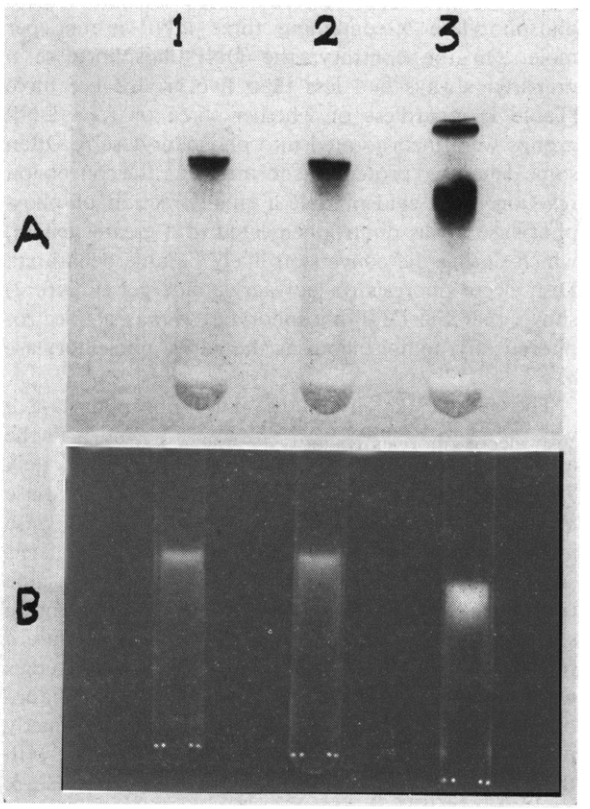

FIGURE 2: Polyacrylamide disc gel electrophoresis of modified and native phosphorylases. (A) Detection of protein: (1) phosphorylase *a;* (2) DNP-phosphorylase *a;* (3) DNP-phosphorylase *b* modified in the presence of AMP and glucose-1-P. (B) Enzymic activity for samples of part A.

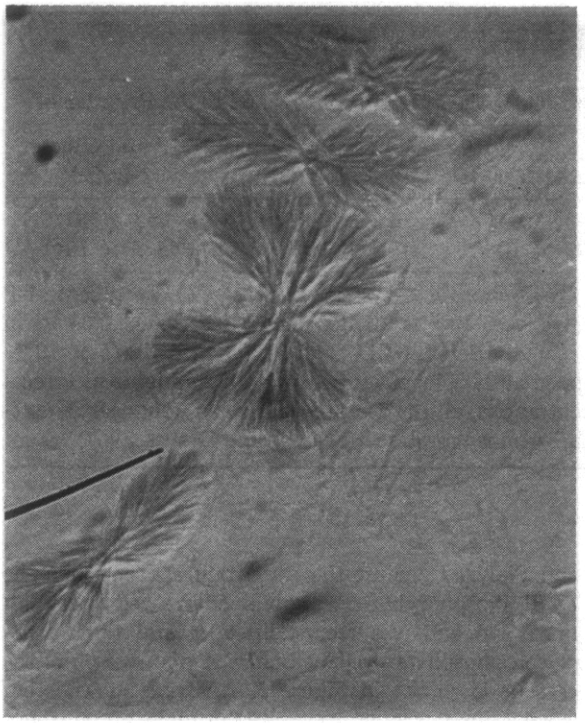

FIGURE 3: DNP-phosphorylase *a* crystals. Picture taken with a 35-mm film with a 440-fold magnification.

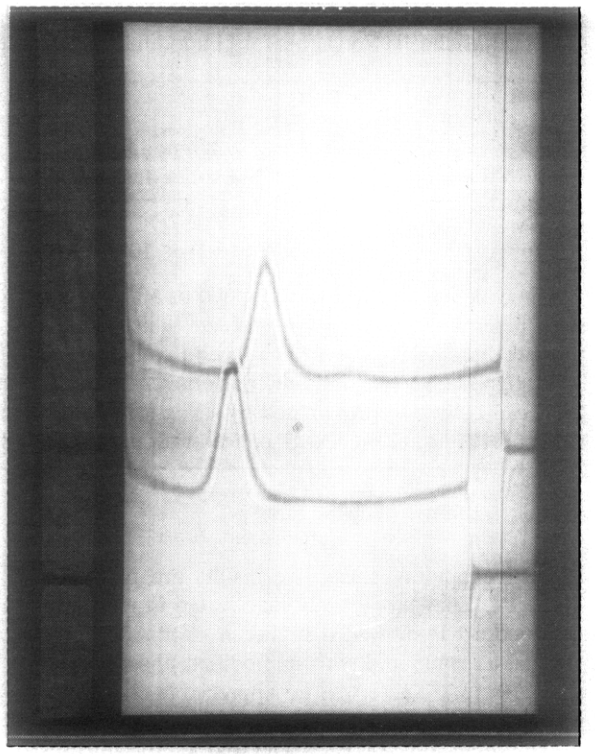

FIGURE 4: Ultracentrifugation of DNP-phosphorylase *a* and phosphorylase *a*. Enzyme (8 mg/ml) was centrifuged at 59,780 rpm at 25° in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8). The picture was taken 30 min after the centrifuge reached a speed of 59,780 rpm. Sedimentation direction is from right to left. Upper curve: DNP-phosphorylase *a;* lower curve: phosphorylase *a*.

of molecular weight, six groups would have been expected to be present in phosphorylase *a* obtained from phosphorylase *b* containing three DNP groups per mole. On the contrary, the DNP-phosphorylase *a* prepared always had less than five groups per mole (Table I) regardless of whether three or four DNP groups were incorporated into phosphorylase *b*. Often some denatured protein was formed after the conversion reaction. It is evident that a small fraction of phosphorylase *b* was dinitrophenylated to a greater extent, which during the conversion likely became denatured. Disc electrophoresis on polyacrylamide gel (Figure 2) shows that the DNP-phosphorylase *a* was as electrophoretically homogeneous as the native phosphorylase *a*.

The specific activity of the DNP-phosphorylase *a* was found to be 1700 Cori units/mg of protein in the absence of AMP, which corresponded to approximately 75–80% of the specific activity of native phosphorylase *a*. The ultracentrifugal behavior of DNP-phosphorylase *a* and phosphorylase *a* is illustrated in Figure 4. Their sedimentation velocities were found to be the same ($s_{20,w}$ = 13.8 S). A small amount of a slower sedimenting species was also detected in the DNP-phosphorylase *a* fraction (upper curve). Separate ultracentrifugal studies with partially converted DNP-phosphorylase (mixture of DNP-phosphorylase *a* and *b* forms) showed distinctly two components which corresponded in $s_{20,w}$ with those for partially converted native phosphorylase *b*. Thus, it was concluded that the slower sedimenting species in Figure 4 was probably DNP-phosphorylase *b*.

The absorption spectrum of DNP-phosphorylase *a* and native phosphorylase *a* are compared in Figure 5. The unmodified phosphorylase *a* shows maximum absorbance at 333 mμ which is due to enzyme-bound pyridoxal phosphate and the DNP derivative exhibits a maximum at 356 mμ. The difference spectrum shows a peak at 362 mμ which compares well with the spectra of amino-substituted DNP-amino acids and peptides (Fraenkel-Conrat *et al.*, 1955).

The Michaelis constant of DNP-phosphorylase *a* for AMP, glucose-1-P, and glycogen were investigated. Figure 6 shows the double-reciprocal plots for glucose-1-P and AMP for DNP-phosphorylase *a*, and the native enzyme. Since phosphorylase *a* is active without AMP, but can be stimulated by its presence in the assay mixture, Δ specific activity is used in the double-reciprocal plot. The ratio of activities −AMP:+AMP was slightly lower for the derivative and hence its higher Δ specific activity. The K_M's for glucose-1-P are 7 and 6×10^{-3} M and the K_M's for AMP are 5 and 3.7×10^{-7} M for modified and native enzymes, respectively. The K_M's for DNP-phosphorylase *a* also support the conclusion drawn in the case of phosphorylase *b* derivatives, that glucose-1-P and AMP preserve their respective binding sites when present during dinitrophenylation.

The results illustrated in Figure 7 show the effect of dinitrophenylation on the K_M for glycogen. In this case, the K_M for glycogen was found to be different with DNP-phosphorylase *a* (0.95 mM glucosyl residues as compared with 0.52 mM with the unmodified enzyme). Since no dinitrophenylation experiments were

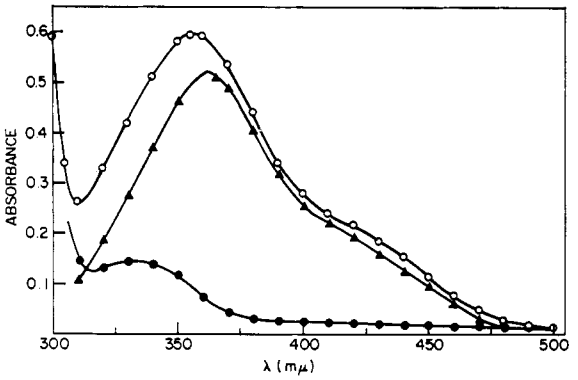

FIGURE 5: Absorption spectrum of DNP-phosphorylase *a* and native phosphorylase *a*. Enzyme (2.5 mg/ml) in 0.04 M glycerophosphate–0.03 M cysteine (pH 6.8). DNP-phosphorylase *a* (○); phosphorylase *a* (●); difference spectrum (▲).

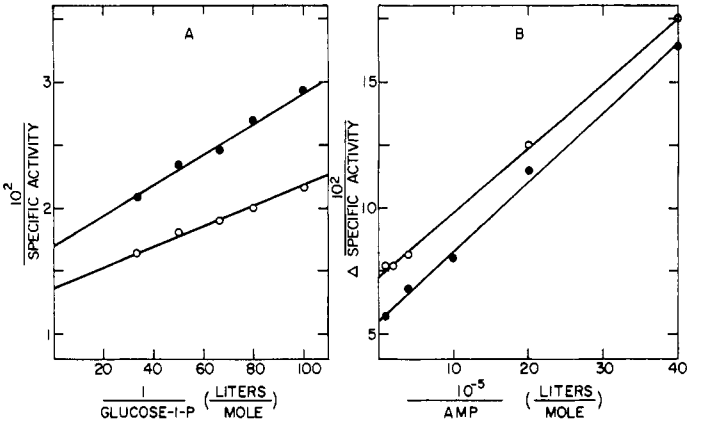

FIGURE 6: Reciprocal plot for glucose-1-P and AMP for DNP-phosphorylase *a* and phosphorylase *a*. Assay mixtures contained: (A) 1% glycogen, 10^{-3} M AMP, and varying concentrations of glucose-1-P; and (B) 1% glycogen, 0.016 M glucose-1-P, and varying concentrations of AMP. DNP-phosphorylase *a* (●) and phosphorylase *a* (○).

carried out in the presence of polysaccharide, it cannot be ascertained whether glycogen could maintain the characteristics of its binding site as observed in the case of dinitrophenylation in the presence of glucose-1-P and AMP.

Properties of Different DNP Derivatives of Phosphorylase. Since dinitrophenylated phosphorylase *b* prepared in the presence of AMP and glucose-1-P could be converted into phosphorylase *a*, the conversion of different DNP-phosphorylase *b* derivatives prepared in the absence and in the presence of substrate or activator alone was attempted. Dinitrophenylation in the absence of AMP and glucose-1-P or in the presence of either one alone was carried out as before using phosphorylase *b* (20 mg/ml) until 45–50% inactivation occurred. The reaction was stopped and the derivatives were converted into the *a* forms as described previously. The DNP derivatives of phosphorylase *a* obtained from this reaction could also be crystallized. The derivative prepared in the absence of glucose-1-P and AMP (75–80% inactivated) showed some change in the ratio of activities (−AMP:+AMP) after incubation with phosphorylase kinase indicating a partial conversion into the *a* form but no crystalline derivative could be obtained.

Discussion

The results reported in this paper deal with dinitrophenylation of phosphorylase *b*, the accompanying changes in its catalytic properties, and with its conversion into DNP-phosphorylase *a*. The enzyme used for this study was dialyzed against 0.025 M Tris buffer. This dialyzed enzyme contained four less titratable SH groups and possessed 70% of the specific activity of the undialyzed enzyme. Incubation with an eightfold molar excess of DNFB resulted in loss of enzymic activity and analyses showed that lysyl and cysteinyl residues were dinitrophenylated.

The studies of Madsen and Cori (1956) showed that phosphorylase may be inactivated by modification of sulfhydryl groups with PMB and that this inactivation can be fully reversed by cysteine. This property was used in this work in order to assess the relative contribution of modification of lysyl and cysteinyl residues by DNFB to enzyme inactivation. If the reacted cysteinyl residues are not important for activity, it might be expected that a PMB derivative of phosphorylase *b* would show the same rate of inactivation as phosphorylase *b*. This was found to be the case; therefore it was concluded that inactivation was due to the modification of lysyl residues. Huang and Madsen (1966) also observed that the rates of carbamylation of native phosphorylase and PMB-treated phosphorylase were identical and concluded similarly that inactivation by cyanate is not due to modification of sulfhydryl groups.

It was found that the presence of glucose-1-P or AMP markedly influenced the rates of inactivation of phosphorylase *b* and the catalytic characteristics of the resulting DNP derivatives. This protective effect is

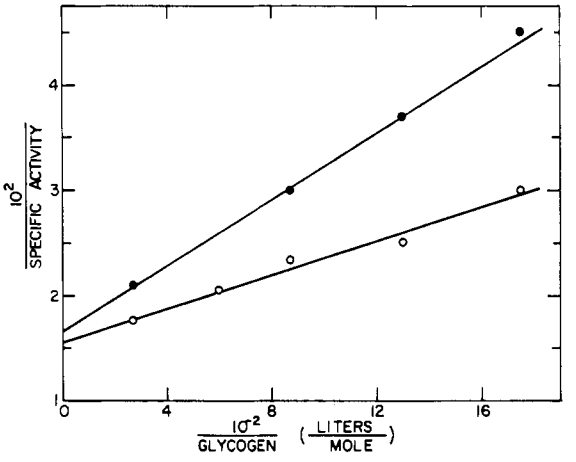

FIGURE 7: Reciprocal plot for glycogen for DNP-phosphorylase *a* and phosphorylase *a*. Assay mixtures contained 0.04 M glucose-1-P and varying concentrations of glycogen. DNP-phosphorylase *a* (●) and phosphorylase *a* (○). Initial velocities were measured from phosphate progress curves by construction of the tangent t_0 with the use of a tangentimeter. Glycogen concentrations are calculated as total glucosyl units.

probably related to maintenance of the structure of binding sites, since changes in Michaelis constants for substrate or activator were observed only when dinitrophenylation was carried out in the absence of substrate or activator, respectively. Moreover, when both substrate and activator were present, there was no change in K_M's for glucose-1-P and AMP. This protection by substrate and activator might suggest that the changes observed in Michaelis constants in their absence is the result of chemical modification of residues at the binding sites. Alternatively, inactivation could result from a conformational change induced by modification of residues distant from the active center. Owing to the apolar nature of the DNP moiety, a new conformation might be induced by a strong interaction of the DNP group with a hydrophobic region; that this binding is in an asymmetric environment is demonstrated in part 2 of this study (Johnson *et al.*, 1968). The protection afforded by glucose-1-P alone is somewhat surprising in view of the recent work of Buc (1967) and Madsen and Shechosky (1967), which suggested that native phosphorylase *b* existed in the inactive (T) state with little or no affinity for substrate or activator. Further work is necessary to determine whether this effect of glucose-1-P is due to binding to some extent to the (T) state at its substrate site or whether this action is related to a binding of glucose-1-P at a different site.

The results reported by Gold (1968) differ markedly from the present work. He observed under his experimental conditions that the major product of dinitrophenylation was DNP-cysteine and not DNP-lysine. This is no doubt due to pretreatment of the enzyme with dithiothreitol followed by gel filtration on Bio-Gel P-10. The fact that several SH groups react rapidly with DNFB (Gold, 1968) and DTNB (Damjanovich and Kleppe, 1966) indicates the possible distribution of these groups near or on the surface of the enzyme. Since dialysis against Tris buffer results in the disappearance of four otherwise titratable sulfhydryl groups, it is likely that these sulfhydryl groups are easily oxidized during dialysis and therefore are not available for reaction with DNFB. It is not surprising that in the two studies there exists differences in protection of enzymic activity by glucose-1-P and AMP and in the spectral properties of the derivatives. If the major product of dinitrophenylation were ε-DNP-lysine, it would be expected that the spectrum of the protein would be comparable with that of ε-DNP-lysyl peptides (λ_{max} 360 mμ). This is the case in this study, whereas in the work of Gold, the absorption spectrum is consistent with that for the modification of cysteinyl residues. The differences in protection by AMP and glucose-1-P by DNFB cannot be directly compared since different groups are modified in these studies. It is possible, however, that slight protection observed by Gold could be a reflection of poor binding since dinitrophenylation was carried out at a higher pH where the enzyme essentially has no catalytic activity.

The difficulty commonly encountered with chemical modification of complex enzymes like phosphorylase is the formation of a population of species with different extents of modification, *e.g.*, Huang and Madsen (1966) observed many protein bands on disc gel electrophoresis of carbamylated phosphorylase. Our results and those of Gold (1968) clearly show that pretreatment of enzyme prior to modification also is a determining factor. The separation of the different protein derivatives is often difficult. In this study, the DNP-phosphorylase *b* obtained was also found to be heterogeneous but after conversion of this derivative into the *a* form followed by crystallization, the DNP-phosphorylase *a* was found essentially homogeneous by ultracentrifugal and electrophoretic studies. The fact that DNP-phosphorylase *a* can be easily purified is a valuable tool for further studies in the elucidation of the various structural aspects of phosphorylase.

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Dinitrophenylation of Glycogen Phosphorylase. II. Circular Dichroism of the Modified Enzyme*

George F. Johnson, George Philip, and Donald J. Graves†

ABSTRACT: Dinitrophenylated glycogen phosphorylase *b* exhibits a new, long-wavelength circular dichroism band in addition to the circular dichroism of the enzyme-bound pyridoxal 5′-phosphate (PLP) reported earlier by Johnson and Graves (Johnson, G. F., and Graves, D. J. (1966), *Biochemistry 5*, 2906). This new circular dichroism ascribed to the dinitrophenyl (DNP) residues disappears in 5 M guanidine hydrochloride, showing that the DNP circular dichroism band depends on protein conformation and is not simply a consequence of the DNP group being covalently bound to an optically active amino acid residue. The circular dichroism band is not affected by either AMP, an activator of phosphorylase *b*, or α-D-glucose 1-phosphate (glucose-1-P), a substrate. Conversion of phosphorylase *b* into *a* gives retention of the circular dichroism, but decreases the rotational strength of the band slightly. Treatment of dinitrophenylated phosphorylase *a* (DNP-*a*) with phosphorylase phosphatase to give dinitrophenylated phosphorylase *b* (DNP-*b*) causes circular dichroism spectrum to revert to that characteristic of the original DNP-*b*. Reaction of *p*-hydroxymercuribenzoate (PMB) with DNP-phosphorylase *b* results in a substantial decrease of the circular dichroism; removal of the PMB from the protein by cysteine returns the circular dichroism to its original magnitude. Removal of PLP from DNP-phosphorylase *b* by hydroxylamine in the presence of an imidazole–citrate buffer causes loss of PLP circular dichroism with only a partial loss of the circular dichroism of the dinitrophenyl residues. The noncovalent interaction of the DNP group with the protein demonstrated by the optical activity of the DNP residues might well mask other neighboring residues or could conceivably mediate a conformation change. Therefore, the change in enzymic activity observed in DNP-phosphorylase by Philip and Graves (Philip, G., and Graves, D. J. (1968), *Biochemistry 7*, 2093 (this issue; paper I)) might result from something more complicated than the chemical modification of ε-amino groups of lysine and SH groups of cysteine.

2,4-Dinitrofluorobenzene (DNFB)[1] and 2,4-dinitrochlorobenzene have been widely and profitably used to chemically modify proteins. In part I of this series (Philip and Graves, 1968), the chemical consequences of dinitrophenylation of glycogen phosphorylase were investigated. We report here that the DNP residues in DNFB-modified phosphorylase are optically active and that this optical activity depends on protein conformation. Circular dichroism of DNP residues in an enzyme, as used here, has possibilities of being a useful, new technique in the better understanding of how incorporation of DNP groups into an enzyme can modify its catalytic activity.

Experimental Section

Materials. Glycogen phosphorylases *b* and *a* and the various DNFB-modified phosphorylases used in this study were prepared as described in part I (Philip and Graves, 1968). Phosphorylase phosphatase was used as a partially purified preparation. The purification was carried through step 1 of Hurd *et al.* (1966). Conversion of phosphorylase *a* into phosphorylase *b* was performed as described by Hurd *et al.* (1966). Guanidine hydro-

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[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: DNFB, 2,4-dinitrofluorobenzene; DNP-b and DNP-*a*, dinitrophenylated phosphorylase *b* and *a*, respectively; PMB, *p*-hydroxymercuribenzoate; PLP, pyridoxal 5′-phosphate.