Sulfhydryl Groups of Rabbit Muscle Glycogen Phosphorylase *b*. Reaction with Dinitrophenylating Agents*

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ABSTRACT: The reaction of glycogen phosphorylase *b* from rabbit muscle with fluorodinitrobenzene (FDNB) and chlorodinitrobenzene (CDNB) was studied by following the absorbance at 335 mμ. Both reagents react rapidly with four sulfhydryl groups and much more slowly with other sulfhydryl, amino, and phenolic hydroxyl groups. The reacting groups were identified from the ultraviolet absorption spectrum of the dinitrophenyl (DNP) protein and by means of the isotope dilution method using ^{14}C-labeled reagents. Substrates and adenosine 5′-monophosphate (AMP) have little inhibitory effect on the reaction of the enzyme with FDNB; however AMP has a distinct, though small, protective effect upon the reactive sulfhydryl groups against CDNB. The reaction of CDNB with the reactive sulfhydryl groups at 30°, pH 8.0, proceeds with a second-order rate constant of *ca.* 60 M^{-1} min^{-1}, while mercaptoethanol and cysteine have corresponding rate constants of 4.7 and 12.3 M^{-1} min^{-1}, respectively. Inactivation of the enzyme by both reagents was demonstrated using an assay that included 1.0×10^{-4} M AMP. AMP, but not glucose 1-phosphate, had a slight protective effect against activity loss with both dinitrophenylating agents.

The major effect of dinitrophenylation upon the enzyme kinetics is to decrease greatly the affinity of the enzyme for AMP and glucose 1-phosphate (other substrates were not investigated) without very large changes in the limiting reaction rate. Dinitrophenylation does not change the sedimentation coefficient of phosphorylase *b*. The four reactive sulfhydryl groups are not essential to the catalytic function of the enzyme or to substrate binding, although they exert a large quantitative effect upon the apparent affinity of enzyme for substrates. The function of these especially reactive groups has not been elucidated.

Although considerable work has been done on the structure, physical properties, and kinetics of rabbit muscle glycogen phosphorylase (α-1,4-glucan:orthophosphate glucosyltransferase EC 2.4.1.1.), little is known about the mechanism of action of the enzyme in terms of the functional groups involved in interactions with substrates. This type of information is frequently obtained by chemically modifying certain functional groups of the enzyme, more or less specifically, and observing the effect of the change upon the structure of the protein and the kinetics of the catalytic reaction. The success of this technique depends upon the specificity and completeness with which a single functional group or set of groups can be modified and the detail in which the kinetics of the enzyme can be studied.

An early study of glycogen phosphorylase along these lines was done by Madsen and Cori (1956), who observed the effect of *p*-mercuribenzoate upon phosphorylases *a* and *b*. The reagent combined with 14 sulfhydryl groups of phosphorylase *a*[1] and caused both forms of the enzyme to dissociate into subunits of apparent mol wt 135,000. Enzymatic activity was completely abolished but all effects could be reversed by addition of a high concentration of cysteine. These observations were extended by Kudo and Shukuya (1964) and Jókay *et al.* (1965). The latter authors found that the rate of inactivation of phosphorylase *b* by *p*-mercuribenzoate and *N*-ethylmaleimide was increased in the presence of glucose 1-phosphate and decreased by AMP. In another study Damjanovich and Kleppe (1966) followed the reaction of phosphorylase *b* with the specific sulfhydryl reagent DTNB[2] (Ellman, 1959); approximately two to three sulfhydryl groups reacted rapidly without apparent change in enzymic activity and another two to three sulfhydryl groups reacted slowly, producing extensive inactivation. The less active sulfhydryl groups were protected against reaction with DTNB in the presence of AMP.

Huang and Madsen (1966) investigated the reaction of phosphorylases *a* and *b* with cyanate. The reagent reacted extensively with ε-amino groups of lysine residues but, contrary to expectation, had no effect upon sulfhydryl groups. No gross changes in enzyme struc-

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[1] All values quoted have been recalculated in terms of the molecular weights recently reevaluated by Seery *et al.* (1967). These are 370,000 for phosphorylase *a* and 185,000 for phosphorylase *b*.

[2] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: FDNB, 2,4-dinitrofluorobenzene; CDNB, 2,4-dinitrochlorobenzene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

ture resulted, but the preparations were very heterogeneous on electrophoresis and the kinetics were complex. Large numbers of amino groups had to be carbamylated to produce significant inactivation.

Much work has been done to produce specific modification of the functional groups associated with the pyridoxal 5′-phosphate bound to phosphorylase. This has been summarized by Fischer and Krebs (1966).

The present study is an attempt to analyze the effect of FDNB and the much less reactive CDNB upon functional groups in rabbit muscle glycogen phosphorylase *b* and upon the activity of the enzyme. It was found that the enzyme contains approximately four sulfhydryl groups that are particularly reactive with the two dinitrophenylating agents, although there are a number of less reactive sulfhydryl, amino, and phenolic hydroxyl groups that undergo reaction at an appreciable rate, especially with FDNB. The major kinetic effect of dinitrophenylation of the four reactive sulfhydryl groups seems to be a large decrease in the affinity of the enzyme for AMP and glucose 1-phosphate (other substrates were not investigated). Because of the complexity of the reaction it was not possible to deduce the precise function of the reactive sulfhydryl groups.

Experimental Procedures

Phosphorylase *b* was prepared by the method of Fischer and Krebs (1962) starting with frozen muscle from New Zealand white rabbits obtained from Pel-Freez. In order to ensure the absence of phosphorylase *a* the defrosted muscle was incubated at 37° for 1 hr prior to grinding. Also, the dialyzed enzyme was incubated at 30° for 1 hr at pH 7.2 prior to the heat treatment at pH 8.8 (E. H. Fischer, personal communication). Mercaptoethanol was substituted for cysteine at all times. Phosphorylase *b* was recrystallized at least four times prior to use. The assay of Illingworth and Cori (1953) was modified by the use of mercaptoethanol and the inclusion of 1 mg/ml of bovine serum albumin. Enzyme activities were of the order of 1500–1600 units/mg, although freshly prepared enzyme occasionally had specific activities as high as 2000 units/mg.

Glucose 1-phosphate dipotassium salt, AMP, and shellfish glycogen were obtained from Sigma Chemical Co. Crystallized bovine serum albumin was purchased from Pentex, Inc., and *S*-DNP-cysteine, ϵ-*N*-DNP-lysine, and *O*-DNP-tyrosine were purchased from Mann Laboratories, Inc. Glucose 1-phosphate was purified by treating a concentrated solution with 0.03 equiv of barium acetate and recrystallizing the supernatant twice from aqueous methanol. Glycogen was freed from nucleotides by treatment in solution with an equal weight of Norit A.

Ultraviolet absorption measurements at single wavelengths were made with a Zeiss PMQ II spectrophotometer which had been calibrated with potassium chromate solution. Spectra were determined with a Cary Model 14 double-beam spectrophotometer.

Crystalline enzyme was prepared for dinitrophenylation by dissolving 20 mg/ml in barbital buffer (0.050 M sodium barbital–1 × 10^{-3} M EDTA, pH 8.0) containing 5 mM dithiothreitol. The solution was passed through a column of Bio-Gel P-10 (Bio-Rad Laboratories) that had been preequilibrated with barbital buffer and the protein concentration of the peak fractions was adjusted to 4.0 mg/ml (or other stated concentration) with the same buffer. Dinitrophenylation was carried out at 30° by adding 0.020 volume of FDNB or CDNB in acetonitrile to the phosphorylase solution which had been preequilibrated at 30°. When necessary, the reaction was quenched with 0.04 volume of 0.30 M mercaptoethanol and dialyzed exhaustively against 0.01 M sodium phosphate–1 × 10^{-4} M EDTA (pH 7.0) in the cold.

Rates of reaction of phosphorylase *b* with FDNB and CDNB were determined by following the absorbance of the reaction mixture at 335 mμ. All runs were done in duplicate and the corresponding points, which agreed closely, were averaged. The absorbance values were corrected for the initial absorbance of protein and reagent, and the extinction coefficient of the protein-bound DNP groups was corrected for the extinction coefficient of the reagent at 335 mμ, since one molecule of reagent disappears for each DNP group incorporated. Both FDNB and CDNB are stable in the absence of protein. Measurements in the presence of substrates were conducted by diluting 0.80 volume of enzyme with 0.20 volume of substrate prior to adding reagent.

Rates of inactivation were determined similarly to dinitrophenylation rates using a modification of the zero-order assay of Hedrick and Fischer (1965). At appropriate time intervals aliquots of the reaction mixture were diluted with a buffer containing 0.10 M sodium maleate, 0.040 M mercaptoethanol, and 1 mg/ml of bovine serum albumin (pH 7.0). Assays were carried out with 0.200 ml of this solution and 0.200 ml of a solution of substrate such that the final concentrations were 0.075 M glucose 1-phosphate, 1.0% glycogen, 1.0 × 10^{-4} M AMP, 0.10 M sodium maleate, 0.020 M mercaptoethanol, and 0.5 mg/ml of bovine serum albumin (pH 7.0). Incubation was 5.0 min at 30° and the reaction was stopped with 5.0 ml of 0.1 N H_2SO_4.

Kinetics of modified enzymes was done by diluting the dinitrophenylation mixture with a buffer containing 0.080 M sodium maleate, 4 × 10^{-4} M EDTA, 1.0 mg/ml of bovine serum albumin, and 0.040 M mercaptoethanol (pH 6.8). This solution was diluted with an equal volume of 2% glycogen and the reactions were started by adding 0.500 ml of enzyme–glycogen solution to 0.500 ml of substrate solution such that the final solution contained the stated concentrations of glucose 1-phosphate and AMP, 0.020 M maleate, 1 × 10^{-4} M EDTA, 0.25 mg/ml of bovine serum albumin, 0.01 M mercaptoethanol, 0.5% glycogen, and enough KCl to give an ionic strength of 0.2 M. Incubations were done at 30° for 10 min and the reactions were stopped as described above.

Proteins were hydrolyzed at 112° for 22 hr with 0.5 ml of glass-distilled constant-boiling HCl in tubes sealed at 50 μ. Amino acid analysis was carried out on a Beckman amino acid analyzer using an accelerated system.

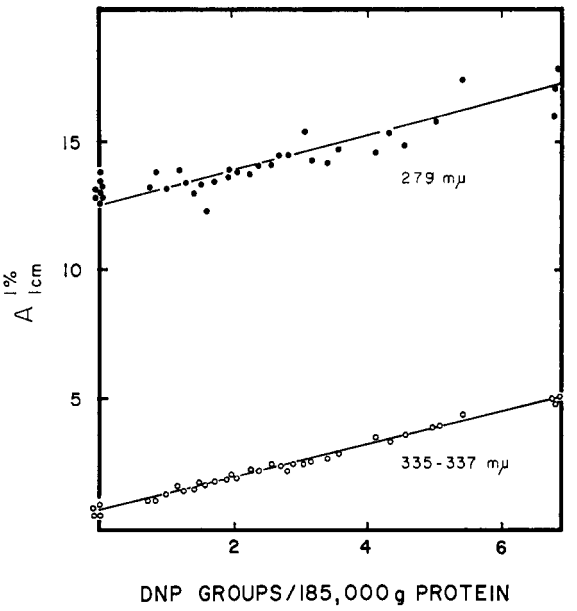

FIGURE 1: Specific absorbancy of DNP phosphorylase *b* Protein concentrations were determined by hydrolysis and amino acid analysis and DNP concentrations by measurement of radioactivity.

Radioactivity measurements were done with a Nuclear-Chicago Mark I liquid scintillation counter. The phosphor solution was prepared with 0.50 ml of aqueous sample, 0.50 ml of Hyamine hydroxide (1 M in methanol), 5.0 ml of absolute ethanol, and 10.0 ml of toluene containing 0.50% 2,5-diphenyloxazole and 0.030% 1,4-bis[2-(5-phenyloxazolyl)]benzene. Efficiencies were determined for each sample by adding 0.100 ml of absolute ethanol containing 1 mg of [7-^{14}C]-benzoic acid of accurately known specific activity (New England Nuclear Corp.) and recounting. Statistical accuracy was 1–2%.

Specific activity and concentration of [U-^{14}C]FDNB (Nuclear-Chicago Corp.) in acetonitrile was assayed by adding an aliquot to 0.05 M NaOH and, after hydrolysis was complete, determining the radioactivity and the absorbance at 360 mμ. Assay of [U-^{14}C]CDNB (New England Nuclear Corp.) was done similarly; however the reagent was converted into *S*-DNP-mercaptoethanol in 0.1 M sodium carbonate containing 0.01 M mercaptoethanol and the absorbance at the 339-mμ maximum was measured. Both reagents were diluted to specific activity of about 0.25 mCi/mmole.

Isotope dilution analysis was carried out by adding 2 μmoles each of *S*-DNP-cysteine, ε-*N*-DNP-lysine, and *O*-DNP-tyrosine to the radioactive DNP protein (*ca.* 1 mg) prior to hydrolysis. Hydrolysis was done as usual but for only 6 hr. The derivatives were reisolated by chromatographing on the 4-cm column of the amino acid analyzer using pH 5.28 buffer and conducting the column effluent to an external fraction collector. The derivatives are well separated and *S*-DNP-cysteine emerges in a peak sharp enough to use directly. Fractions containing each of the other derivatives were combined and concentrated by acidifying to pH 2.2 with HCl and readsorbing on a 0.7 × 1.0 cm column containing Beckman type AA-27 resin. After first washing the column with distilled water, the derivatives were eluted rapidly with 0.1 N NH_3. The products were evaporated to dryness and taken up in a small volume of pH 5.28 buffer.

Radioactivity was determined as described above. Concentrations of *S*-DNP-cysteine and ε-*N*-DNP-lysine were estimated from their absorbance at 335 and 362 mμ, respectively; the concentration of *O*-DNP-tyrosine could be determined with the amino acid analyzer since it emerges later than arginine.

Ultracentrifugal studies were carried out with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics and an RTIC unit for temperature control. Runs were done at 16° at a rotor speed of 47,660 rpm using a pair of 12-mm single-sector Kel F cells, one of which had a +1° wedge window.

Results

Extinction Coefficient of Phosphorylase b. Velick and Wicks (1951) in their work on the amino acid composition of phosphorylase *a*, found a specific absorbancy, $A^{1\%}_{1cm}$, of 11.7 at 279 mμ, based upon the dry weight of the protein. In a more recent study of the amino acid composition of phosphorylase *b*, Appleman *et al.* (1963) determined a value of 11.9 for the same constant, again based upon dry weight of protein. Because of its importance to this study, the extinction coefficient was redetermined by making use of the amino acid analysis provided by Appleman *et al.* This analysis is based upon their finding that the weight of a sample of phosphorylase *b* can be satisfactorily accounted for as the sum of the weights of the constituent amino acid residues (aside from two residues of pyridoxal phosphate).

Samples of phosphorylase *b* were dialyzed against 0.01 M sodium phosphate or glycerophosphate (pH 7), their absorbance was determined, and aliquots were hydrolyzed for amino acid analysis. The molar concentration of protein was calculated from the content of each of the acidic and neutral amino acids, other than proline and half-cystine, and the values were averaged. Average deviations were usually about 3%. However, the average of the calculated specific absorbancies in eight independent determinations was 13.1 ± 0.3 at 279 mμ. This value was used throughout the present work. No explanation can be offered for the difference at this time.

Ultraviolet Absorption of DNP Protein. Phosphorylase *b* was allowed to react with 5×10^{-4} M [U-^{14}C]-FDNB under standard conditions at pH 8.0 and aliquots were removed periodically and treated with mercaptoethanol to destroy the remaining reagent. These samples, after dialysis, were used to determine the absorption spectrum of DNP-phosphorylase *b*, the concentration of DNP groups through measurement of radioactivity, and the protein concentration by hydrolysis and amino acid analysis.

DNP proteins prepared in this way contained up to seven DNP groups per molecule; the spectra, as expected, consisted of two absorption maxima, one due

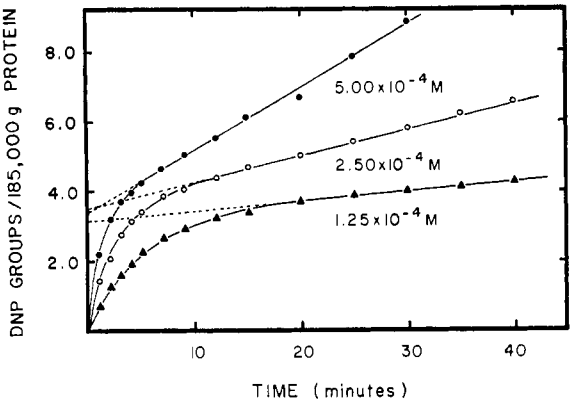

FIGURE 2: Time course of the reaction of phosphorylase *b* with FDNB. At pH 8.0, 30°, 3.72 mg/ml of protein, variable FDNB concentration. The absorbance at 335 mμ was followed and an empirical extinction coefficient of 11,900 was used to calculate the concentration of DNP groups incorporated.

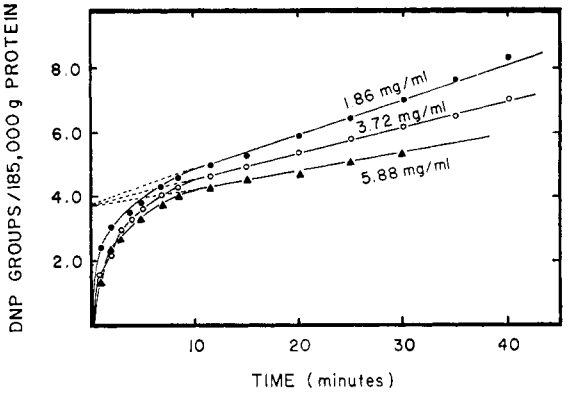

FIGURE 3: Time course of the reaction of phosphorylase *b* with FDNB. At 2.50 × 10^{-4} M FDNB, variable protein concentration. See Figure 2 for other conditions.

primarily to the protein and the other to the DNP groups. Native unmodified phosphorylase *b* has a maximum at 279 mμ and a weak maximum at 333 mμ resulting from the enzyme-bound pyridoxal phosphate (Kent *et al.*, 1958). All the dinitrophenylated samples retained the maximum at 279 mμ and showed a relatively stronger maximum at longer wavelength. The maximum absorption ranged from 335 to 337 mμ in the samples containing the highest numbers of DNP groups. This indicates that initially primarily sulfhydryl groups react with FDNB. The maximum absorption of *S*-DNP-mercaptoethanol is at 339 mμ (ε 11,200), while that of *S*-DNP-cysteine is reported to occur at 330 mμ (ε 9600) in 0.2 N acetic acid (Mahowald, 1965). The absorption maximum of ε-*N*-DNP-lysine lies at 362 mμ.

Specific absorbances are given in Figure 1. The absorbancies at the long-wavelength maximum give a good line which is described by the eq

$$A^{1\%}_{1\,cm} = 0.703 + 0.643N \qquad (1)$$

where N is the number of DNP residues in the protein molecule. This is equivalent to an average molar extinction coefficient of 11,900 for the protein-bound DNP groups. The specific absorbancy at the short-wavelength maximum (279 mμ) shows considerable scatter when plotted against N; the best line calculated by the least-squares method is

$$A^{1\%}_{1\,cm} = 12.55 + 0.700N \qquad (2)$$

This agrees within experimental error with the average value of 13.1 found for the unmodified enzyme. The higher value is preferred for determining concentrations of native enzyme. It is possible to estimate the concentration and DNP content of any sample prepared by the method described above by the use of eq 1 and 2.

When phosphorylase *b* is dinitrophenylated with CDNB the long-wavelength absorption maximum remains at 335 mμ, indicating almost exclusive reaction with sulfhydryl groups; this is to be expected in view of the lower reactivity of the reagent. Because of the similarity of the groups dinitrophenylated (see below) the specific absorbancies for phosphorylase modified with CDNB wll be assumed to be identical with those obtained with the FDNB-modified enzyme.

Reaction of Phosphorylase b with FDNB and CDNB. The rate of reaction of the enzyme with FDNB can be observed directly by following the absorbance of the solution at 335 mμ. Figure 2 indicates the course of the reaction at three concentrations of FDNB and Figure 3 shows the effect of different protein concentrations upon the reaction. This data can be interpreted in terms of a model in which the protein contains two classes of groups reactive with FDNB: a set of approximately four groups which reacts rapidly and es-essentially completely within 5–20 min and another set which reacts more slowly at a rate which is almost constant over the time of the experiment. The number of rapidly reacting groups is given by the intercept of the extrapolated linear portion of the curve and the rate of reaction of the slowly reacting set is given by the slope.

All the curves have intercepts corresponding to 3.1–3.5 DNP groups/molecule of enzyme. Since the protein consists of two, presumably identical, subunits the rapidly reacting set probably contains four groups. The slopes are not proportional to the initial FDNB concentration but this is partially due to the fact that at low FDNB concentration and at high protein concentration a significant fraction of the FDNB is consumed in the early part of the reaction.

The effect of AMP and substrates upon the reaction with FDNB is indicated in Table I. AMP appears to have a slight protective effect upon the slowly reacting groups while glucose 1-phosphate has the opposite effect of slightly increasing the rate of reaction of these groups. The combination of AMP and glucose 1-phosphate, and an equilibrium mixture of these substances with glycogen and inorganic phosphate, has no net effect upon the slow reaction but seems to reduce the apparent number of rapidly reacting groups. In the last case this may not be significant since there is some difficulty in correcting the initial absorbance for the light-scattering effect of glycogen.

TABLE I: Reaction of Phosphorylase *b* with FDNB in the Presence of AMP and Substrates.[a]

Substrate Added				
AMP (1.0×10^{-3} M)	Glucose-1-P(K_2) (0.020 M)	Glycogen (0.4%) and P (Na_2) (0.020 M)	No. of Rapidly Reacting Groups	Rate of Reaction of Slowly Reacting Groups (DNP groups/min)
−	−	−	3.2	0.064
+	−	−	3.0	0.048
−	+	−	3.4	0.077
+	+	−	2.8	0.060
+	−	+[b]	2.5	0.063

[a] 30°, pH 8.0, 2.50×10^{-4} M FDNB and 4.0 mg/ml of protein. [b] This is an equilibrium mixture of substrates during the reaction.

TABLE III: Second-Order Rate Constants for Reaction of Phosphorylase *b* with CDNB in the Presence of AMP and Substrates.[a]

Substrate Added			
AMP (1.0×10^{-3} M)	Glucose-1-P (K_2) (0.020 M)	Glycogen (0.4%) + P (Na_2) (0.020 M)	k (M^{-1} min^{-1})
−	−	−	62
+	−	−	43
−	+	−	60
+	+	−	42
+	−	+[b]	42

[a] 30°, pH 8.0, 5.00×10^{-4} M CDNB and 4.0 mg/ml of protein. [b] This is an equilibrium mixture of substrates during the reaction.

Chlorodinitrobenzene, as expected, reacts much more slowly with the protein than does FDNB. Figures 4 and 5 show the course of the reaction at three concentrations of CDNB and three protein concentrations, respectively. If the assumption is made that there are 3.5 groups that react at approximately the same rate it is possible to treat the reaction as a second-order process and apply the appropriate rate equation

$$\frac{1}{b-a} \ln \frac{a(b-x)}{b(a-x)} = kt \qquad (3)$$

where a is the initial concentration of reactive groups, b is the initial concentration of CDNB, and x is the concentration of dinitrophenylated groups. A plot of log $[(b-x)/(a-x)]$ *vs.* time for the data of Figure 4 is shown in Figure 6. Slight adjustments of the number of reactive groups assumed per molecule of enzyme improves the linearity in some instances but no curve requires the assumption of more than 3.8 groups and the value of 3.5 gives the best over-all fit. The curvature is probably due to the fact that the groups which react slowly with FDNB also react at a significant rate with CDNB and this has a pronounced effect on the curve at longer times. The isotope dilution experiment reported below supports this idea. In all cases the curves are linear until at least 2.3 DNP groups have been introduced into the protein molecule.

TABLE II: Second-Order Rate Constants for Reaction of Phosphorylase *b* with CDNB under Various Conditions.[a]

CDNB ($M \times 10^4$)	Protein (mg/ml)	k (M^{-1} min^{-1})
10.0	4.07	51
5.00	4.07	58
2.50	4.07	56
5.00	1.98	68
5.00	3.97	57
5.00	5.95	53

[a] 30°, pH 8.0.

Table II gives the second-order rate constants derived from the data in Figures 4 and 5. Clearly, the data cannot be reconciled with a simple second-order reaction since the derived constants are not invariant when different initial concentrations of reactants are used. The decrease in rate constant with increasing protein concentration is reminiscent of the observations of Huang and Madsen (1966) who found a similar effect in the inactivation of phosphorylase *a* by cyanate. These authors have proposed a possible explanation for the phenomenon. Although the reaction is not a simple second-order process, the second-order rate constant is a useful parameter for comparing the rates of reaction under different conditions when the initial concentrations of enzyme and CDNB are kept constant.

Substrates appear to have little influence on the reaction while AMP exerts a slight inhibitory effect; the results are given in Table III as second-order rate constants. Those reactions in which AMP is omitted have approximately the same rate while all the reactions including AMP have the same, slightly lower, rate.

For comparison, second-order rate constants were determined for the reaction of CDNB with mercaptoethanol and cysteine under the conditions used for phosphorylase *b*. Approximately 0.8×10^{-4} M CDNB was allowed to react with 10, 20, and 30 mM mercaptoethanol at 30° and the absorbance was followed at 339 mμ until no further change occurred. The reaction showed good pseudo-first-order kinetics and a second-order constant of 4.7 ± 0.2 M^{-1} min^{-1} could be calculated. The measurements with cysteine were done simi-

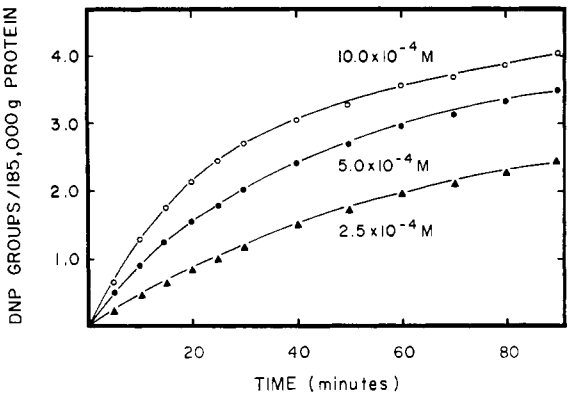

FIGURE 4: Time course of the reaction of phosphorylase *b* with CDNB. With 4.07 mg/ml of protein, variable CDNB concentration. See Figure 2 for other conditions.

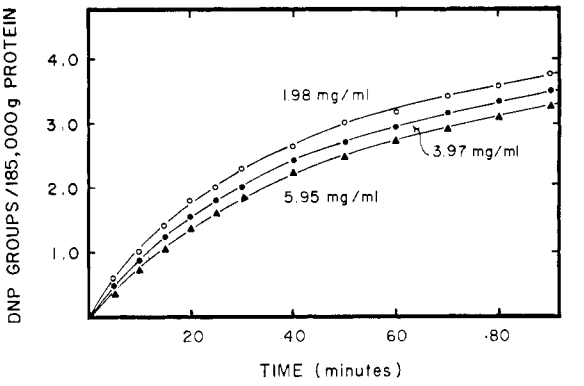

FIGURE 5: Time course of the reaction of phosphorylase *b* with CDNB. With 5.00 × 10^{-4} M CDNB, variable protein concentration. See Figure 2 for other conditions.

larly, using 5, 10, and 15 mM cysteine and following the reaction at 335 mμ. The second-order rate constant was calculated to be 12.3 ± 0.1 M^{-1} min^{-1}; however, the true value is probably lower since the distinct yellow color of the reaction mixture indicated that some reaction occurred with amino groups of cysteine.

Inactivation of Phosphorylase b with FDNB and CDNB. When an attempt was made to follow the decline of enzyme activity during dinitrophenylation of phosphorylase *b* using the zero-order assay of Hedrick and Fischer (1965) the rate of inactivation in the presence of 2.5 × 10^{-4} M FDNB was found to be very slow. Similarly, little inactivation could be observed in the presence of 5.0 × 10^{-4} M CDNB. This is attributable to the fact that, as will be seen later, the effect of dinitrophenylation is mainly upon the affinity of the enzyme for AMP and glucose 1-phosphate rather than upon the maximum velocity of the reaction. The concentrations of AMP and substrates in the assay are high and the only effects that can be observed are changes in maximum velocity and extremely large changes in binding.

Inactivation could be demonstrated readily when the assay was modified by using 1.0 × 10^{-4} M AMP at pH 7.0. Figure 7 shows the inactivation of phosphorylase *b* in the presence of 2.5 × 10^{-4} M FDNB. The major loss in activity occurs during the first 5 min; in this time the enzyme has taken up 3.4 DNP groups, of which 3.1 are from the rapidly reacting set and 0.3 is from the slowly reacting set. When the reaction is carried out in the presence of AMP or substrate there is a distinct protection by AMP either alone or in the presence of glucose 1-phosphate or an equilibrium mixture of substrates. Glucose 1-phosphate alone had only a slight protective effect.

Inactivation by 5.0 × 10^{-4} M CDNB is illustrated in Figure 8; the process is much slower than with FDNB and the activity falls to 36% in 1 hr. In this time *ca.* 2.7 DNP groups are incorporated in the protein. Glucose phosphate appears to have little influence upon inactivation while AMP has a small protective effect, either alone or with glucose 1-phosphate or an equilibrium mixture of substrates.

Kinetics of Dinitrophenylated Phosphorylase b. The dependence of the initial velocity of the catalytic reaction upon concentrations of AMP and glucose 1-phosphate was determined for native and dinitrophenylated phosphorylase *b*. In all cases the rate depends upon the glucose 1-phosphate concentration according to the classical Michaelis–Menten equation

$$v = V_{max}S/(K_M + S) \quad (4)$$

The dependence of rate upon the AMP concentration is better described by the modified Hill equation (Changeux, 1963)

$$\log \frac{V_{max} - v}{v} = \log K - n \log S \quad (5)$$

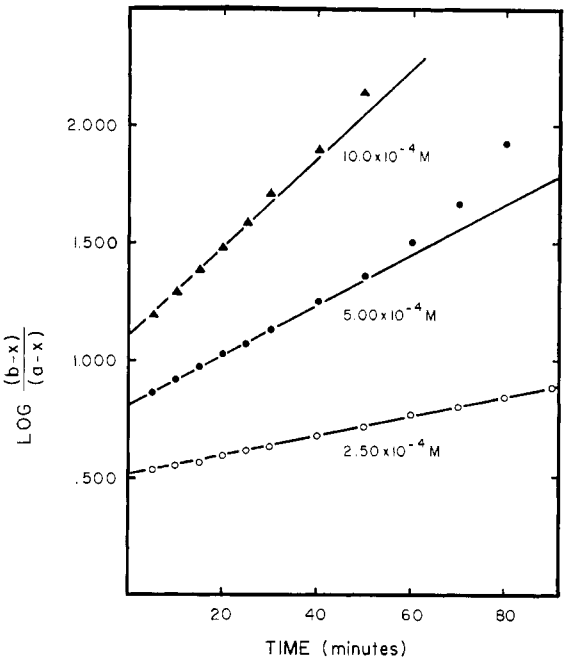

FIGURE 6: Time course of the reaction of phosphorylase *b* with CDNB treated as a second-order reaction. With 4.07 mg/ml of protein, variable CDNB concentration. See Figure 2 for other conditions.

TABLE IV: Kinetics of Native and Dinitrophenylated Phosphorylase *b*.[a]

Dinitrophenylating Agent	DNP Groups	Glucose-1-P		AMP		
		V_{max} (μmoles min^{-1} mg^{-1})	K_M (M $\times$ 10^3)	V_{max} (μmoles min^{-1} mg^{-1})	n	K ($M_n \times 10^6$)
	0	75	2.3	65	1.6	0.21
FDNB	3.1	59	10.9	54	1.4	8.5
FDNB	5.0	46	42	27	1.2	130
	0	69	1.5	64	1.6	0.20
CDNB	1.1	68	3.6	53	1.6	0.55
CDNB	3.4	55	13.3	53	1.4	8.0

[a] Initial velocities were calculated as micromoles of inorganic phosphate liberated per minute per milligram of enzyme under the conditions given in the Experimental Section. Substrate concentrations were calculated as the mean of the initial and final concentrations. Initial glucose 1-phosphate concentrations were 40, 25, 15, 10, 7, and 5 mM at 1.0 mM AMP, while the AMP concentrations were 100, 20, 10, 6, 4, and 3 $\times$ 10^{-5} M at 25 mM glucose 1-phosphate.

where the empirical constant n is a measure of the degree of cooperativity between AMP binding sites. Data from representative experiments are presented in Table IV. Inactivation by both dinitrophenylating agents is marked by relatively small changes in V_{max} and much larger changes in K_M (for glucose 1-phosphate) or K (for AMP). Values for K are very sensitive to the value of n selected for a particular set of data. In the experiments of Table IV n tends to decrease with increasing dinitrophenylation; however, in other experiments n remained *ca.* 1.5 and it is not possible to conclude that dinitrophenylation results in changes in n.

Identification of Reactive Groups. Samples of phosphorylase *b*, dinitrophenylated to varying extents with [^{14}C]FDNB and [^{14}C]CDNB, were hydrolyzed in the presence of known amounts of *S*-DNP-cysteine, ϵ-*N*-DNP-lysine, and *O*-DNP-tyrosine. The carriers were reisolated and their specific activities were determined. With this information it is possible to calculate the number of DNP groups that have coupled with the sulfhydryl groups of cysteine residues, the amino groups of

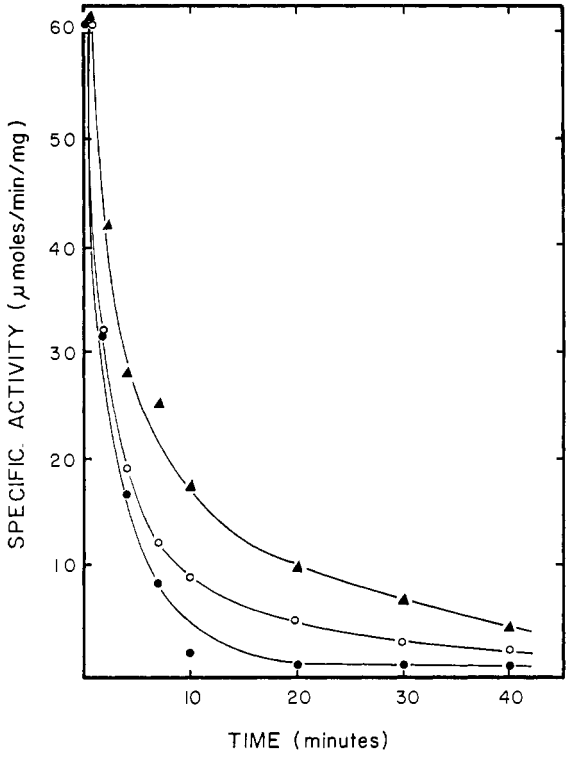

FIGURE 7: Inactivation of phosphorylase *b* by FDNB. pH 8.0, 30°, 2.5 $\times$ 10^{-4} M FDNB and 4.0 mg/ml of protein. Activity assay is done with 0.075 M glucose 1-phosphate, 1% glycogen, and 1.0 $\times$ 10^{-4} M AMP. (●) No addition; (○) 20 $\times$ 10^{-3} M glucose 1-phosphate; (▲) 1.0 $\times$ 10^{-3} M AMP.

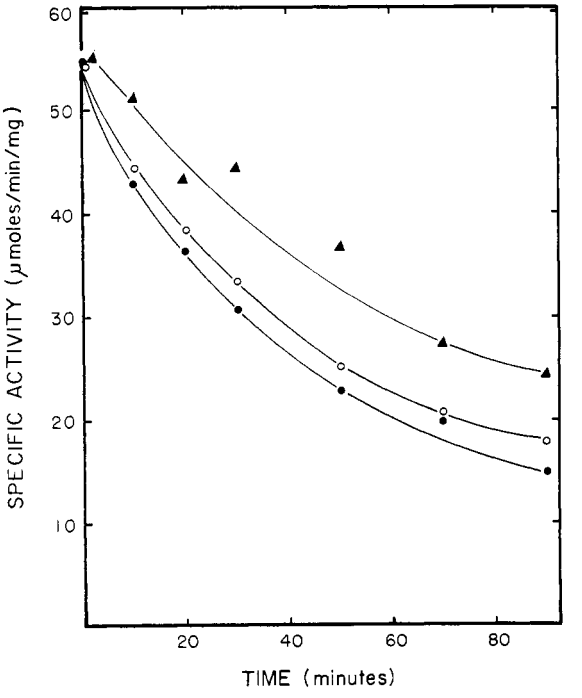

FIGURE 8: Inactivation of phosphorylase *b* by CDNB. With 5.0 $\times$ 10^{-4} M CDNB. See Figure 7 for other conditions.

TABLE V: Identification of Dinitrophenylated Functional Groups in Phosphorylase *b*.

Dinitrophenylating Agent	Total DNP Groups/Molecule of Protein	*S*-DNP-cysteine	ε-*N*-DNP-lysine	*O*-DNP-tyrosine
FDNB	3.1	2.6	0.44	0.12
FDNB	3.8	3.4	0.55	0.17
FDNB	8.4	6.4	1.5	1.0
CDNB	1.8	1.9	0.22	0.07
CDNB	3.5	3.2	0.34	0.14

TABLE VI: Sedimentation Coefficients of Native and Dinitrophenylated Phosphorylase *b*.

Dinitrophenylating Agent	DNP Groups/Molecule of Protein	Protein Concn (mg/ml)	Sedimentation Coefficient[a] (S)
None	0	3.8	7.51
FDNB	5.0	3.8	7.82
None	0	3.8	7.65
CDNB	3.4	3.8	7.87

[a] No corrections were made for solvent or temperature. See text for details.

lysine residues, and the phenolic hydroxyl groups of tyrosine residues. The results are presented in Table V. For each sample the sum of the DNP-amino acids slightly exceeds the total number of DNP groups in the protein; the only obvious cause is that significant amounts of one of the carriers might have been destroyed during the acid hydrolysis more rapidly than the corresponding DNP-amino acid residue in peptide linkage within the partially hydrolyzed polypeptide chain. The over-all recovery of both *S*-DNP-cysteine and ε-*N*-DNP-lysine was between 60 and 80%, while that of *O*-DNP-tyrosine varied between 20 and 70%.

Clearly the sulfhydryl groups of cysteine residues are the major reactive species with both reagents, as predicted from the ultraviolet absorption of the DNP proteins. FDNB begins to react significantly with amino groups and phenolic hydroxyl groups only after large numbers of DNP groups have been introduced. The selectivity of CDNB is even greater than that of FDNB, but it too reacts with some amino and phenolic groups. This similarity in the pattern of reaction suggests that the major difference between the two reagents is one of reaction rate and not a matter of the kind of functional group reacting.

Other types of functional groups that might react with dinitrophenylating agents are α-amino and the side chain of histidine. It is likely that phosphorylase *b* does not contain a free α-amino group, since many attempts to identify it have failed (Appleman *et al.*, 1963). Possibly some imidazole-DNP-histidine residues were present in the protein; they would not have been detected since no carrier was available. However, in view of the high total recovery of ^{14}C in the form of the three amino acids investigated, it is unlikely that any large fraction of the DNP groups could be on histidine residues.

Sedimentation Velocity of Modified Phosphorylase b. In order to determine whether dinitrophenylation has any effect upon the degree of association of the enzyme subunits, sedimentation velocity measurements were made with modified enzyme. In one experiment a sample of enzyme was treated with 2.5×10^{-4} M FDNB for 20 min, long enough to introduce 5.0 DNP groups, and then quenched with mercaptoethanol. A similar sample was treated in the same way but the FDNB was omitted. These two solutions were then run simultaneously in the ultracentrifuge at 16°. Table VI indicates the result of this and a similar experiment in which the enzyme was treated with 5.0×10^{-4} M CDNB for 90 min. The uncorrected sedimentation coefficients suggest that there is no important change in the degree of association as a result of dinitrophenylation. This does not bear upon possible changes in the degree of association of the enzyme under the conditions of the activity assay where the protein is present at a concentration of *ca.* 0.03 mg/ml at 30°. The ultracentrifuge patterns gave no indication of major inhomogeneity in any of the samples.

Effect of Other Conditions upon the Dinitrophenylation Reaction. In work published simultaneously with this study Philip and Graves (1968) report quite different results for the reaction of phosphorylase *b* with FDNB. Their experiments indicate that amino groups are the major reactive species and that large protective effects upon enzymic activity are observed when the reaction is conducted in the presence of AMP or glucose 1-phosphate. The main difference in the experimental method is that Philip and Graves prepared the enzyme for dinitrophenylation by exhaustive dialysis against 0.025 M Tris buffer at pH 7.6 and carried out dinitrophenylation in the same buffer. In order to reconcile these apparently conflicting results the enzyme was prepared according to this procedure and dinitrophenylation was followed spectrophotometrically at 335 mμ, 30°, as described in the Experimental Section. The rapid phase of the dinitrophenylation was significantly slower and the intercept was much reduced. At a concentration of 2.5×10^{-4} M FDNB and 3.4 mg/ml of protein the intercept was 1.1 DNP/mole (assuming the same extinction coefficient observed previously) and the slow phase proceeded at a rate of 0.036 DNP/min. The ultraviolet spectrum of the protein, after 40-min reaction with FDNB, showed a broad maximum with a peak near 338 mμ. In a second experiment done under similar conditions using 5.0 mg/ml of protein the intercept was 0.9 DNP/mole and the slow phase had a rate of 0.029 DNP/min. Another experi-

ment was conducted according to the procedures used in this work but substituting 0.025 M Tris (pH 7.6) for 0.050 M barbital (pH 8.0). The other aspects, such as inclusion of EDTA, dithiothreitol, and gel filtration, were as usual. This preparation, on dinitrophenylation at a protein concentration of 4.5 mg/ml, gave an intercept of 3.1 DNP/mole and a slow phase with a rate of 0.038 DNP/min. This suggests that the observed differences are due to destruction or blocking of reactive sulfhydryl groups when the enzyme is treated by exhaustive dialysis in the absence of protective agents.

Discussion

Madsen and Cori (1956), in their work on the reaction of rabbit muscle phosphorylase with *p*-mercuribenzoate, detected approximately 14 sulfhydryl groups in phosphorylase *a* and indicated the presence of 7 sulfhydryl groups in phosphorylase *b*.[1] Their observation that some of these sulfhydryl groups reacted more rapidly than others was confirmed by Kudo and Shukuya (1964) who found that seven sulfhydryl groups of phosphorylase *a* are significantly more reactive with *p*-mercuribenzoate than are the remaining sulfhydryls. Although it is not always safe to extrapolate the chemical behavior of one form of the enzyme to the other form, this is consistent with the present finding that four sulfhydryl groups of phosphorylase *b* are particularly reactive with FDNB and CDNB. The high reactivity of *ca.* four sulfhydryl groups of phosphorylase *b* was also observed by Damjanovich and Kleppe (1966) with DTNB at pH 6.8.

Philip and Graves (1968) reported that FDNB reacts predominantly with amino groups of phosphorylase *b* under conditions similar to those used in this study. The enzyme used in their work was prepared for dinitrophenylation by exhaustive dialysis without a protective agent such as EDTA. In order to reconcile these results similar experiments were attempted and it was found that the concentration of reactive sulfhydryl groups is greatly reduced when the enzyme is treated in this way. It is possible that the sulfhydryl groups undergo oxidation to disulfides or react with traces of metals; modifications such as these would protect the sulfhydryls from dinitrophenylation but would be easily reversible under the conditions of the enzyme assay, which is done in the presence of excess mercaptan. Thus, the differences in the effects observed by Philip and Graves is probably due to the fact that they modify functional groups other than the reactive sulfhydryls; these may be some of the slowly reacting groups observed in this study.

In view of the varying reactivity of different sulfhydryl groups in phosphorylase it is interesting to note that the total sulfhydryl content of phosphorylase *b* has been reported to be 15, determined by means of DTNB and *p*-mercuribenzoate titration in the presence of denaturing agents (Damjanovich and Kleppe, 1967). Similar experiments with DTNB in 6.7 M urea solution carried out with enzyme prepared according to the procedure outlined in this work indicated 16 sulfhydryl groups. Thus, half of the sulfhydryl groups of the enzyme are unreactive with *p*-mercuribenzoate in the absence of a denaturing agent.

Although the protein sulfhydryl groups are considerably more reactive with CDNB than is either mercaptoethanol or cysteine, having a second-order rate constant of *ca.* 60 M^{-1} min^{-1} compared with 4.7 M^{-1} min^{-1} for mercaptoethanol and 12.3 M^{-1} min^{-1} for cysteine, the high reactivity is not too great to be explained on the basis of differences in degree of ionization. At pH 8.0 about 22% of the sulfhydryl groups of cysteine are ionized, almost exclusively in the form of $^{-}SCH_2CH(NH_3^{+})CO_2^{-}$ (Benesch and Benesch, 1955), and this leads to a pH-independent rate constant of 56 M^{-1} min^{-1}. Mercaptoethanol has a pK_a of 9.5 (Edsall and Wyman, 1958) and a pH-independent rate constant of *ca.* 150 M^{-1} min^{-1}. If the protein sulfhydryl groups have low pK_a's, owing to the nearness of positively charged groups or dipoles, they would be largely ionized at pH 8.0 and the observed rate constant would be near the pH-independent rate constant.

There is some difficulty in attributing the effect of dinitrophenylation on the enzyme kinetics to modification of any particular functional group, since the reaction is not highly specific with either dinitrophenylating agent. Little doubt exists that substitution of the reactive sulfhydryl groups is responsible for observed changes in K_M for glucose 1-phosphate and K for AMP, because these changes occur before any significant dinitrophenylation of other functional groups has taken place. However it is not clear whether the decrease in V_{max} is due to substitution of reactive sulfhydryls or to substitution of less reactive sulfhydryl, amino, or phenolic hydroxyl groups. The total change observed in V_{max} is not large and it could be caused by partial modification of some critical group in the enzyme. Clearly, the reactive sulfhydryl groups have no obligatory role in the catalytic mechanism of the enzyme; extensive substitution of those groups leaves the enzyme with nearly full activity at high substrate concentration.

The reactive sulfhydryls could conceivably function in several ways. (i) They could be directly involved in substrate binding, although their participation cannot be obligatory. Since there is probably an interaction between AMP and glucose 1-phosphate binding sites (Madsen, 1964) the sulfhydryl groups could be involved at either or both sites; however, the fact that some protection against dinitrophenylation is observed in the presence of AMP and little is found with glucose 1-phosphate suggests that the sulfhydryls are most likely involved in the binding of AMP. (ii) An alternative to this idea is that the sulfhydryls have no direct function in binding ligands, but that their substitution with bulky dinitrophenyl groups inhibits binding by steric hindrance. (iii) A third possibility is that the sulfhydryl groups are not located at the binding sites but are involved in the mechanism by which the protein mediates allosteric interactions between the binding sites (Monod *et al.*, 1963). In the absence of specific information about this mechanism it is impossible to predict what effects should be expected when the sulfhydryls are modified.

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