Philip, G., and Graves, D. J. (1967), *Federation Proc. 26*, 601.
Pontremoli, S., Luppis, B., Traniello, S., Wood, W. A., and Horecker, B. L. (1965), *J. Biol. Chem. 240*, 3469.
Porter, R. R. (1957), *Methods Enzymol. 4*, 221.
Rowley, P. T., Tchola, O., and Horecker, B. L. (1964), *Arch. Biochem. Biophys. 107*, 305.
Sealock, R. W., and Graves, D. J. (1967), *Biochemistry 6*, 201.
Shaltiel, S. (1967), *Biochem. Biophys. Res. Commun. 29*, 178.
Velick, S. F., and Wicks, L. F. (1951), *J. Biol. Chem. 190*, 741.

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-

* From the Department of Biochemistry and Biophysics, Iowa State University, Ames, Iowa 50010. *Received September 25, 1967.* Journal Paper J-5790 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa, Project No. 1510. This research was supported by a research grant (GM-09587) from the National Institutes of Health, U. S. Public Health Service.

† Recipient of a research career development award of the U. S. Public Health Service (Grant GM6753).

[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.

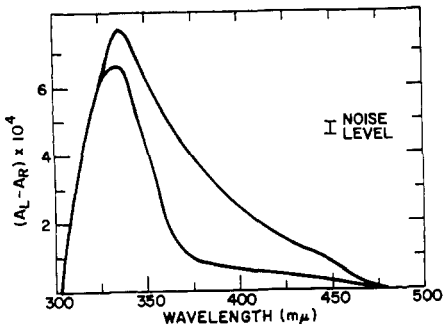

FIGURE 1: Circular dichroism of DNP-phosphorylase *b* and native *b*. Native phosphorylase *b* (lower curve) and DNP-*b* (upper curve) are 10 mg/ml in 0.05 M β-mercaptoethanol–0.04 M β-glycerophosphate (pH 6.8). DNP-*b* was prepared in the presence of AMP (2×10^{-3} M) and glucose-1-P (0.032 M).

chloride was purchased from Eastman Organic Chemicals and imidazole from Sigma Chemical Co. All other chemicals were described in part I of this series.

Methods. Circular dichroism spectra were taken with a Jouan dichrograph, modified as previously described by Martin *et al.* (1966), but with replacement of the original ADP-modulating crystal with one of ammonium dideuterium phosphate, which reduced the modulating voltage by one-half. Spectra were taken in 1-cm semimicro silica cells. Maximal absorbance in the region studied (500–300 mμ) was 2.0. Unless otherwise indicated, all protein concentrations used were 10 mg/ml; concentrations were determined as described in part I of this work.

Results

Johnson and Graves (1966) and Torchinsky *et al.* (1965) have shown that bound pyridoxal phosphate in phosphorylase is optically active. The circular dichroism spectrum of phosphorylase *b* can be seen in Figure 1 (lower curve). By comparing this spectrum with a dinitrophenylated sample of phosphorylase *b* at the same concentration (upper curve), we see that new optical activity has appeared in the long-wavelength region. Conversion of the DNP-*b* into the *a* form does not destroy this new optical activity as can be seen from Figure 2. The new circular dichroism can best be visualized by subtracting the circular dichroism of the native enzyme from that of the DNP enzyme to yield a difference curve. The curves obtained by this procedure are those shown in Figure 3.

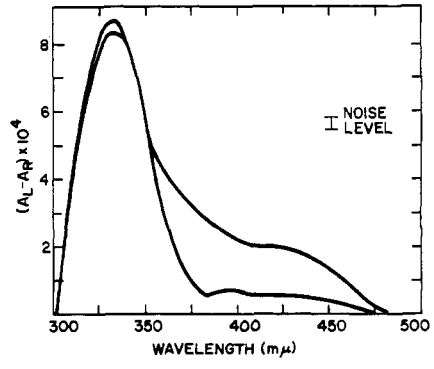

FIGURE 2: Circular dichroism of DNP-phosphorylase *a* and native *a*. Native phosphorylase *a* (lower curve) and DNP-*a* (upper curve) are 10 mg/ml in 0.03 M cysteine–0.04 M β-glycerophosphate (pH 6.8). DNP-*a* was prepared from phosphorylase *b*, dinitrophenylated in the presence of AMP (2×10^{-3} M) and glucose-1-P (0.032 M).

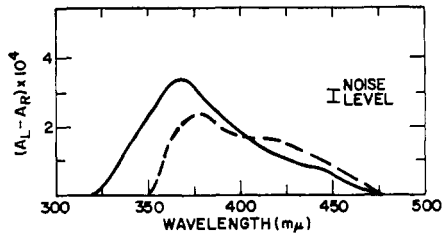

FIGURE 3: Difference circular dichroism between native and DNP-phosphorylases. Solid line represents the difference circular dichroism between native *b* and DNP-*b* (shown in Figure 1), and the dashed line, that between native *a* and DNP-*a* (shown in Figure 2).

Both difference curves are positive and have maxima outside the region of bound pyridoxal phosphate. The absorption spectrum of DNP-*a* (Philip and Graves, 1968) has a maximum at 362 mμ with a shoulder at approximately 430 mμ. The difference circular dichroism curves in Figure 3 also show maxima in this region with shoulders at the longer wavelengths. Experimentally, determination of the exact maxima are difficult, but the observed maxima are consistent with the absorption spectrum of the DNP enzyme. Since both cysteinyl and lysyl residues were modified, the optical activity observed in the difference curves could result from either or both of these DNP residues. From the shapes and maxima of the difference curves, dinitrophenylation seems to have no direct effect on bound PLP circular dichroism as determined from the circular dichroism difference curves. The phosphorylase *b* difference curve is the same regardless of whether the enzyme was dinitrophenylated in the presence of glucose-1-P, AMP, or both of these together.

Conversion of the *b* form of the enzyme into the *a* form causes changes in both magnitude and shape of the difference curve. In the preceding paper, it was observed that conversion of DNP-*b* into DNP-*a* was not quantitative. We found that the average number of dinitrophenyl residues in the protein had decreased after conversion. Probably the highly dinitrophenylated species were removed in this process. This could explain the decreased rotational strength observed in the DNP-*a* enzyme. However, the difference observed between DNP-*a* and -*b* circular dichroism could also reflect different environments about the dinitrophenyl groups in the two enzymes. A test of this possibility was made by preparing DNP-*a* and then treating with phosphorylase phosphatase to obtain DNP-*b*. This procedure allows comparison of DNP-*b* and of the same homogeneity and dinitrophenyl group content. The results can be seen in Figure 4. The DNP-*b* produced shows a circular dichroism spectrum quite similar to the DNP-*b* in Figure 1. The differences between DNP-*b* and -*a* circular dichroism are therefore of an

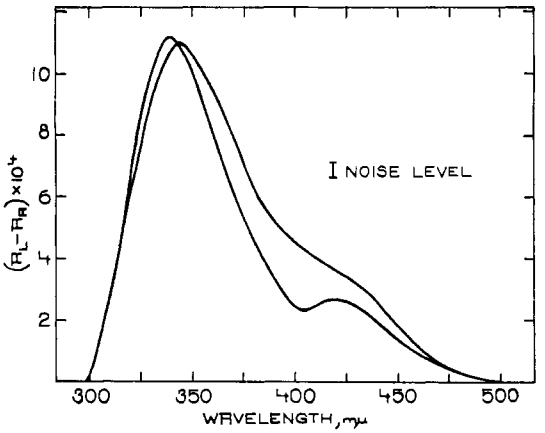

FIGURE 4: Circular dichroism of DNP-*a* (lower curve) and of DNP-*b* (upper curve) prepared from DNP-*a* with phosphorylase phosphatase, DNP-*a* and DNP-*b* (10 mg/ml) in 0.05 M Tris–0.03 M mercaptoethanol (pH 7.3). The ratio of activities (−AMP/+AMP) were 0.70 and 0.10 for DNP-*a* and DNP-*b*, respectively, as measured according to Illingworth and Cori (1953).

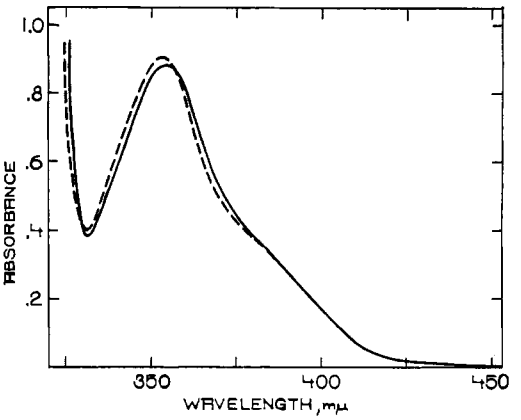

FIGURE 5: Absorption spectra of DNP-*a* (solid line) and that of DNP-*b* (dashed line) prepared from DNP-*a* with phosphorylase phosphatase. Conditions as in Figure 4 except that DNP-*a* and DNP-*b* were 4 mg/ml.

intrinsic nature. Differences can also be seen in the absorption spectra of the dinitrophenyl groups in the two enzymes. Upon conversion of DNP-*a* into DNP-*b*, a definite blue shift can be seen in the maximum near 360 mμ. The spectra can be seen in Figure 5.

The optical activity of the DNP enzymes can be abolished by making them 5 M in guanidine hydrochloride. The resulting traces coincide with the base line within the instrumental noise level. This indicates that the circular dichroism of the DNP residues depends on the conformational integrity of the enzyme and is not simply a result of being covalently bound to an optically active amino acid residue.

Since the circular dichroism of the DNP residues seems dependent upon the conformation of the enzyme, a study of the effect of substrates on the DNP enzymes was made to see if substrate-induced conformational changes could be observed. AMP (10^{-3} M) and glucose-1-P (0.016 M) had no effect on DNP-*b* circular dichroism; also, AMP (10^{-3} M) and glycogen (0.01%) had no effect. A complete equilibrium system was not used because problems with precipitation made the results erratic.

The reaction of excess PMB with phosphorylase *b* is accompanied by inactivation and dissociation to a monomeric species (Madsen and Cori, 1956). Reaction of the DNP-*b* with excess PMB also leads to complete inactivation and, presumably, dissociation. Figure 6 indicates the effect of PMB on the circular dichroism of native *b*. Reactivation of the PMB enzyme with cysteine leads to a return of the circular dichroism characteristic of the native enzyme (conditions are given in the legend of Figure 6). The effect of PMB, therefore, on the circular dichroism of *b* is reversed along with the enzymic activity. The figure shows that 15–20% of the circular dichroism of the native enzyme is lost reversibly with PMB inactivation. This is in good agreement with the observations of Torchinsky *et al.* (1967). Figure 7 shows the same experiment with DNP-*b* (conditions given in the legend). PMB inactivation causes substantial changes in the circular dichroism of the DNP residues which are reversed on treatment with cysteine. Return of the enzyme activity and the dinitrophenyl circular dichroism again indicate an essentially reversible process as is the case with the native enzyme.

PLP can be resolved from phosphorylase *b* by the procedure of Shaltiel *et al.* (1966), which involves using a deforming agent and a nucleophile that will attack PLP. Incubation of DNP-*b* (prepared in the presence of glucose-1-P and AMP) with 0.28 M imidazole (neutralized to pH 6.8 with sodium citrate) and 0.1 M hydroxylamine hydrochloride (pH 6.8) results in almost complete loss of the circular dichroism due to bound PLP and a substantial loss of circular dichroism due to the DNP residues as illustrated in Figure 8. A comparison of Figure 8 with Figure 1 shows that while only 7% of the original circular dichroism is retained at 330 mμ, 28% of the original circular dichroism at 400 mμ is retained after the resolution process. The PMB and resolution experiments further indicate the dependence of the circular dichroism of DNP residues on enzyme conformation or state of aggregation.

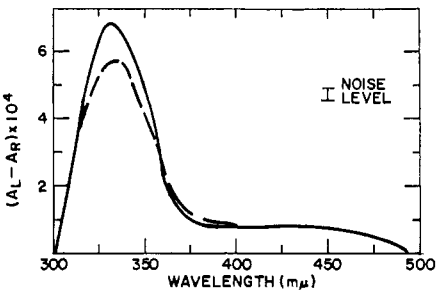

FIGURE 6: Effect of PMB circular dichroism of phosphorylase *b*. Dashed line is phosphorylase *b* (10 mg/ml) in 0.02 M β-glycerophosphate (pH 6.8) allowed to react with a 12 times molar excess of PMB. The residual enzymic activity was 5% of the original. Solid line is the above treated with 0.03 M cysteine (pH 6.8). The extent of reactivation was 95%. Concentration of enzyme is normalized to 10 mg/ml.

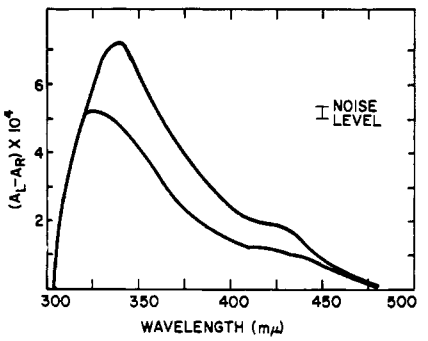

FIGURE 7: Effect of PMB on circular dichroism of DNP-phosphorylase *b*. Lower curve is DNP-phosphorylase *b* (10 mg/ml), in 0.02 M β-glycerophosphate (pH 6.8), dinitrophenylated in the presence of AMP (2×10^{-3} M) and glucose-1-P (0.032 M) and allowed to react with a 12 times molar excess of PMB. The residual enzymic activity was 5% of the initial activity. Upper curve is the PMB enzyme treated with 0.03 M cysteine (pH 6.8). The extent of reactivation was 95%.

Discussion

The magnitude of optical activity assignable to the DNP residues in DNFB-modified phosphorylase is complicated by the presence of a long-wavelength band in the native enzyme (Figures 1 and 2). This small band was not previously noticed in an earlier work (Johnson and Graves, 1966), but instrumental improvements have now indicated its presence. Circular dichroism of the long-wavelength region in the native enzyme at high protein concentration show a small band centered at 425 mμ in addition to the previously reported 333-mμ circular dichroism. If native phosphorylase *b* is resolved of PLP under the condition described for the DNP-*b* in the results section, both the 333- and the 425-mμ circular dichroism bands disappear at the same rate. The resolution of DNP-*b*, however, showed almost complete loss of the 333-mμ circular dichroism, but resulted in an incomplete loss of the long-wavelength circular dichroism. This observation coupled with the observed maxima in the difference circular dichroism spectra (Figure 3) strongly suggests that the circular dichroism observed is connected mainly with the DNP residues and does not result from a perturbation of the native 425-mμ band. From the data presented here, it is not possible to determine to what extent the DNP residues (cysteinyl and lysyl) contributed individually to the observed difference circular dichroism. The differences between DNP-*a* and DNP-*b* circular dichroism were shown by the experiments with phosphorylase phosphatase to be of an intrinsic nature. The difference in environment of the DNP residues indicated by both the circular dichroism and the visible and ultraviolet spectra of the two forms could arise from either subunit aggregation or conformational differences. With the present data, meaningful distinctions between these possibilities cannot be made.

The abolishment of the difference circular dichroism by guanidine hydrochloride indicates that the observed circular dichroism is mediated by the enzyme conformation. This induced optical activity can be interpreted to mean a significant noncovalent interaction of the DNP group with the enzyme. In this respect, Eisen and Suskind (1964) have postulated that the noncovalent free energy of binding of ε-DNP-lysine to its specific antibody population might be attributed to a site involving strong hydrogen bonding of the nitro group, facilitated by the extrusion of interstitial water caused by the nonpolar nature of the DNP group itself. The presence of even a weak noncovalent binding site for DNFB could significantly enhance the rate at which certain residues would be modified relative to others. That circular dichroism appears early with few total cysteinyl and lysyl residues modified lends support to the idea of a noncovalent binding site for DNFB and the resulting DNP group.

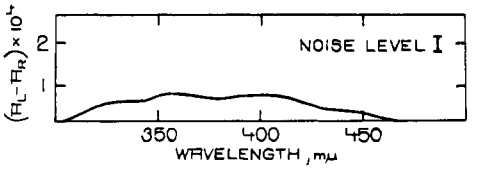

FIGURE 8: DNP-*b* (10 mg/ml) was incubated for 12 hr at 25° in 0.28 M imidazole which had been adjusted to pH 6.8 by addition of solid citric acid. The resolving reagent was 0.1 M hydroxylamine.

Significant changes in the circular dichroism of the DNP groups were observed in both the reaction of DNP-*b* with PMB and the resolution of PLP from DNP-*b*. Under the conditions of both experiments, native phosphorylase *b* is a monomer (Madsen and Cori, 1956; Shaltiel *et al.*, 1966) and presumably DNP-phosphorylase *b* is also a monomer. The results are consistent with either a conformational change in the enzyme that destroys the DNP group interaction with the enzyme surface or, perhaps, a binding site that involves both subunits.

The appearance of optical activity in the absorption band of a DNP group that has been incorporated into an enzyme has certain implications for the technique of chemical modification. It is often assumed that in certain cases, chemical modification of an enzyme (for instance, with DNFB) merely *masks* the residues modified. If a change in enzymic activity is observed, it is further assumed that this group is important for catalytic activity due to direct participation in the catalytic act or through participation in the binding of substrates. Since circular dichroism in the absorption band of a DNP residue implies direct interaction of the DNP group with the enzyme, a change in enzymic activity could also result from a conformational change induced by a DNP group or by noncovalently binding with other groups important for the catalytic activity.

Modification of phosphorylase *b* with DNFB in the presence and absence of both AMP and glucose-1-P results in DNP phosphorylases that differ quite dramatically in their kinetic properties (Philip and Graves, 1968), but these modified phosphorylases are, within experimental error, identical in their circular dichroism spectra. This observation indicates that the DNP residues that show optical activity are probably not residues at the active center of the enzyme. DNP-phosphorylase

a even though recrystallized several times still shows a specific activity less than that of the native enzyme. Since the apparent K_M's for AMP and glucose-1-P remain constant and the K_M for glycogen changes only slightly, the difference in specific activities observed must primarily be a result of a change in maximal velocity. Results of this type might be expected on modification of residues critical for enzyme conformation, or again, if noncovalent interaction of the DNP group itself with the enzyme could cause loss of catalytic activity.

Huang and Madsen (1966) reported that inactivation of phosphorylase *b* by cyanate could be explained by the carbamylation of 23 lysyl residues, whereas Philip and Graves (1968) found that dinitrophenylation of only 4–5 residues (lysyl and cysteinyl) was required for inactivation. Since blocking of the SH groups in phosphorylase by PMB resulted in the same time course of inactivation as found with the native enzyme, it was concluded that only modification of the lysyl residues resulted in inactivation under the conditions used. These contrasting results could be explained by postulating that DNFB is more selective than cyanate in its modification of lysyl residues or that interaction of the DNP group with the enzyme in some manner causes inactivation. These two mechanisms for the inactivation with DNFB are, as previously discussed, consistent with a noncovalent interaction of the DNP group with the protein, which is indicated in the modified phosphorylase by the circular dichroism results. Circular dichroism of the DNP group can thus provide information valuable in cases where only a few of the total residues are modified.

References

Eisen, H. N., and Suskind, G. W. (1964), *Biochemistry 3*, 996.

Huang, C., and Madsen, N. B. (1966), *Biochemistry 5*, 116.

Hurd, S. S., Novoa, W. B., Hickenbottom, J. P., and Fischer, E. H. (1966), *Methods Enzymol. 8*, 546.

Illingworth, B., and Cori, G. T. (1953), *Biochem. Prepn. 3*, 1.

Johnson, G. F., and Graves, D. J. (1966), *Biochemistry 5*, 2906.

Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.

Martin, D. S., Foss, J. G., McCarville, M. E., Tucker, M. A., and Kassman, A. J. (1966), *Inorg. Chem. 5*, 491.

Philip, G., and Graves, D. J. (1968), *Biochemistry 7*, 2093 (this issue; paper I).

Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108.

Torchinsky, Y. M., Livanova, N. B., and Pikhelgas, V. Y. (1965), *Biochim. Biophys. Acta 110*, 619.

Torchinsky, Y. M., Livanova, N. B., and Pikhelgas, V. Y. (1967), *Molekul. Biol. 1*, 28.