The Sulfhydryl Groups of Muscle Phosphorylase

III. IDENTIFICATION OF CYSTEINYL PEPTIDES RELATED TO FUNCTION*

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SUMMARY

Iodoacetamide inactivates glycogen phosphorylase and causes it to dissociate into monomers of 92,500 molecular weight. Three -SH groups on each monomer are alkylated, but the first reacts as rapidly as do model compounds and its alkylation does not cause inactivation. The remaining two -SH groups are alkylated several hundred times slower, at rates comparable to the loss of activity.

The three -SH groups were labeled with 14C-iodoacetamide. The radioactive peptides were isolated from pepsin digests and characterized sufficiently to identify them with certain of the cysteinyl peptides previously isolated and sequenced. The -SH group whose alkylation does not affect activity has been tentatively assigned to the sequence Gly-Cys-Arg-Asp (peptide B). The sequences of the other two cysteinyl peptides which are alkylated concomitantly with the loss of activity are Ala-Cys-Ala-Phe (peptide N) and Asn-Ala-Cys-Asp (peptide A). Another six -SH groups per monomer are not readily alkylated in the native enzyme.

Paper electrophoresis of pepsin digests of the 14C-carboxamidomethyl peptides permits one to compare the kinetics of alkylation of the various peptides with the rates of inactivation. For phosphorylase b, the rate of alkylation of the N peptide is similar to the rate of inactivation and suggests that the alkylation of only this peptide causes loss of activity and dissociation of monomers. Peptide A is alkylated at 50% of the rate of peptide N. For phosphorylase a, the sum of the rates of alkylation of both peptides is similar to the rate of inactivation, and suggests that the alkylation of either results in an inactive enzyme. Alternative interpretations are possible, however, and are discussed. The presence of AMP prevents the alkylation of the peptide A of phosphorylase a, so that in this circumstance the activity loss can be assigned solely to the alkylation of the -SH group of peptide N.

The first paper in this series presented evidence that there are nine -SH groups in each monomer unit of glycogen phosphorylase of molecular weight 92,500 (1). We showed that, of these nine -SH groups, the first one will react with iodoacetamide or other reagents at a rate which is at least as fast as that of model compounds and without the loss of enzymatic activity. The next two -SH groups in each monomer react very much more slowly with iodoacetamide, and their alkylation is correlated with a loss of enzymatic activity and a dissociation of the original dimer of phosphorylase b or tetramer of phosphorylase a. The remaining six -SH groups on each monomer are so well protected by the tertiary structure of the protein that, in our hands, they are not readily available to various reagents until denaturing agents are used.

In a subsequent report from this laboratory, the sequences of the amino acids adjacent to each of the nine -SH groups were presented by Zarkadas, Smillie, and Madsen (2). Since the peptides containing each -SH group could now be isolated from pepsin digests and identified with a known sequence, it became possible to determine which of these peptides contained the -SH groups responsible for the structure-function relationships discussed above. 14C-Iodoacetamide was the reagent of choice for these studies because reasonable rates of inactivation could be obtained under pseudo first order conditions. Reactions could be stopped at any time by the addition of mercaptoethanol and the covalently bound carboxamidomethyl group remains fixed to its original -SH group through subsequent digestion with pepsin and isolation of the peptides. The radioactive peptides could be readily separated by one- or two-dimensional paper electrophoresis, and the extent of reaction quantitatively determined by measuring the radioactivity. Other popular -SH reagents such as CMB1 or DTNB had various obvious drawbacks.

This method made it possible to compare the rates of reaction of the -SH group of each peptide with iodoacetamide to the rate of enzymatic inactivation and its dissociation of the protein. From a comparison of these rates we were able to discern a distinct difference in the mechanism of the inactivation of phosphorylase a as compared to that of phosphorylase b. Furthermore, the kinetic analysis of the data suggested that the alkylation of only one specific -SH group on each monomer unit sufficed to inactivate the enzyme, although the other -SH group may be attacked at the same time. A preliminary experi-

1 The abbreviations used are: CMB, p-chloromercuribenzoic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).
ment confirmed this prediction by showing that the presence of AMP completely protected one of the $-SH$ groups from alkylation but did not prevent the alkylation of the other, and the inactivation.

**METHODS**

Rabbit liver glycogen, purchased from Sigma, was purified by passage through a Dowex 1-X1 column. Glucose 1-phosphate, AMP, and iodoacetamide were also purchased from Sigma. Iodoacetamide-1-14C (5 mCi per mmole) was purchased from the Radiochemical Centre, Amersham. It was dissolved in hot water and crystallized with an excess of nonradioactive material.

Phosphorylase activity was measured in the direction of glycogen synthesis according to Cori, Cori, and Green (3), with 0.02 m sodium $\beta$-glycerophosphate-1.5 mM EDTA, pH 6.8, as the buffer to dilute the enzyme to the appropriate protein concentration. Mercaptoethanol was added to this buffer to a final concentration of 0.05% when necessary to stop the iodoacetamide reaction. The substrate for the reaction was 0.016 M glucose 1-phosphate, 1% glycogen, and 1 mM AMP. Phosphorylase a activities were determined in the presence and absence of AMP.

Crystalline rabbit muscle phosphorylase b was prepared as described by Fischer and Krebs (4) and crystalline phosphorylase a was prepared from phosphorylase b with kinase prepared by the method of Krebs et al. (5), except that mercaptoethanol replaced cysteine in both procedures. The enzymes were recrystallized at least three times. Immediately before use, phosphorylase b was passed through a Sephadex G-25 gel filtration column previously equilibrated with the appropriate buffer. Phosphorylase a crystals were washed several times with cold buffer. Protein concentration was determined from the absorbance at 280 $\mu$m, with a value for $E_{280}$ of 13.2 (6). The method of Lowry et al. (7) was used whenever reagents interfered with the spectrophotometric determination.

The reaction with iodoacetamide was carried out in a buffer containing 0.13 M KCl, 0.033 M sodium glycerophosphate, 1.5 mM EDTA at pH 7.5. The final concentration of phosphorylase b was 9 to 10 mg per ml; of phosphorylase a, 6 mg per ml; and of iodoacetamide, 10 mM, unless otherwise specified. The temperature was maintained at 30° and the protein was always preincubated for at least 20 min. Iodoacetamide solutions were prepared just before the beginning of the experiment. Incorporation into the protein was determined as described previously (1). For peptide analysis, a small volume of the KCl, glycerophosphate, EDTA buffer, containing sufficient mercaptoethanol to give a 50-fold excess over iodoacetamide, was added to the sample. About 15 min later sufficient formic acid was added to yield a concentration of 5%, and the sample was transferred to 8/32 Visking tubing for dialysis against three changes of 5% phosphate, EDTA buffer, containing sufficient mercaptoethanol to give a 50-fold excess over iodoacetamide, was added to the sample. About 15 min later sufficient formic acid was added to yield a concentration of 5%, and the sample was transferred to 8/32 Visking tubing for dialysis against three changes of 5% formic acid over a 36-hour period. The protein concentration was checked and sufficient pepsin added to give a ratio of 1:10 by weight of pepsin to phosphorylase. Digestion was carried out for 20 to 24 hours at 37°. For analysis, 0.6 mg of digested protein was applied as a 1-cm spot on Whatman No. 1 paper and subjected to electrophoresis. For preparative work, 2.4 mg per cm were used on Whatman No. 3MM paper. The methods used for high voltage electrophoresis and NH$_2$-terminal determination were reported previously (8). Electrophoresis strips were analyzed for radioactivity with a Nuclear-Chicago Actigraph III strip scanner with 4$\pi$ geometry.

Mercaptoethanol was added in a 50-fold excess to samples for ultracentrifugation. The Beckman-Spinco model E analytical ultracentrifuge was operated at a speed of 59,780 rpm and a temperature of 20° for the study of dissociation. The percentages of molecular species were determined by estimation of empirically resolved components. The areas so determined were corrected for the Johnson-Ogston effect by the calculation described by Trautman et al. (9) and Schachman (10).

**RESULTS**

**Effect of Iodoacetamide on Structure and Activity of Phosphorylase b—**Iodoacetamide was found to react with the same number of $-SH$ groups in the native protein as does CMB (1). Since the reaction is much slower, a large excess of reagent is required. By using 10 mM iodoacetamide at 30° and pH 7.5, 85% of the activity of phosphorylase b (10 mg per ml) was lost in about 8 hours. A pseudo-first order plot of the activity loss is shown in Fig. 1. The reaction is linear to beyond 95% of completion, a result which is highly reproducible. This reaction is faster and more extensive than that observed with iodoacetic acid, a result usually observed with these two reagents (11).

As occurs with CMB (12), the reaction of iodoacetamide results in a conversion to monomer. Mercaptoethanol was used to stop the reaction at various times and the precipitate which forms with time was centrifuged out. In later experiments, this was found not to be necessary if ultracentrifugation proceeded immediately. The percentage of various components resolved in the analytical ultracentrifuge was determined by area analysis. The 32-min pictures are shown in Fig. 2. It is obvious that, as inactivation by iodoacetamide proceeds, there is a progressive conversion of phosphorylase b dimers, with an average $s_0$ of 8.6, to monomers with an average $s_0$ of 5.3. In Fig. 3b the...
Effect of Iodoacetamide on Activity and Structure of Phosphorylase a—The inactivation of phosphorylase a by iodoacetamide also followed pseudo-first order kinetics but the rate was considerably faster, with an average rate constant of $11.6 \pm 1.0 \times 10^{-2} \text{ min}^{-1}$ for two experiments in which the protein concentration was 6 mg per ml and the iodoacetamide was 10 mm. In one experiment in which 8.7 mg per ml of protein were used, the rate constant was $12.2 \times 10^{-2} \text{ min}^{-1}$. In contrast, phosphorylase b at a concentration of 9 to 10 mg per ml with 10 mM iodoacetamide was inactivated with a rate constant of $3.5 \pm 0.2 \times 10^{-2} \text{ min}^{-1}$ (average of five experiments).

The inactivation of phosphorylase a by iodoacetamide was also accompanied by a dissociation into monomers. However, in this case, the extent of dissociation at each time of sampling during the reaction appeared to lag behind the extent of inactivation, as is illustrated in Fig. 3a.

Isolation and Identification of Peptides Containing Reactive \(\text{-SH}\) Groups—Since approximately 6 moles of iodoacetamide were incorporated per mole of phosphorylase b dimer (1), this suggested that 3 cysteine residues per monomer were reacting. It was possible to identify the peptides containing each particular \(\text{-SH}\) group by scanning a ninhydrin-stained pH 6.5 electropherogram for radioactivity. Such an electropherogram along with its radioactivity scan is shown in Fig. 4, where the positions of the major peptides (A, N, B1, and B2) as well as several minor peptides (C, D, and E) are marked.

A sample (800 mg) of phosphorylase b was alkylated with iodoacetamide for 8 hours. To the 85% inactivated enzyme, which had partially precipitated, mercaptoethanol was added. After dialysis against 5% formic acid to remove the excess reagent the protein was digested with pepsin. The peptide mixture was then separated on paper by high voltage electrophoresis at pH 6.5. The radioactive sections were cut out and further purified by electrophoresis at pH 1.8 and 3.5. The position of the radioactive peptides was determined after each step by scanning side strips. The purified peptides were then eluted from the paper and subjected to amino acid analysis.

Two radioactive peptides were readily obtained by this method and the results of the amino acid analyses are shown in Table I. The yields of carboxymethylcysteine were low in these analyses, a result usually obtained with peptides eluted from paper, and the amounts of carboxamidomethylcysteine were calculated from...
the radioactivity of each peptide. Peptide N, which occurred at the neutral position upon pH 6.5 electrophoresis, contained 2 eq of alanine, 1 of carboxamidomethylcysteine, and 1 of phenylalanine. Analysis indicated that alanine was the NH₂-terminal amino acid. On this basis the peptide was matched with one of the —SH-containing peptides whose sequence had been determined by Zarkadas et al. (2)

Peptide N Ala-Cys-Ala-Phe

The second peptide isolated by this method was denoted A because it is acidic at pH 6.5. It contained 2 residues of aspartic acid, 1 residue of alanine, and 1 of carboxamidomethylcysteine. By the Dansyl-Edman method (13) the NH₂-terminal sequence was established as Asx-Ala. The data for this peptide match those for one of the peptides whose sequence had been previously established (2)

Peptide A Asp-NH₂-Ala-Asp

The radioactive area containing the basic peptides, B₁ and B₂ (Fig. 4), has not been amenable to purification, for adequate characterization and amino acid analysis, by high voltage electrophoresis. The purification of these peptides by other methods is currently in progress. However, from a trypsin digest of the partially purified peptides, a basic radioactive fragment has been isolated which has a composition (Table I) and electrophoretic mobility consistent with a sequence previously elucidated (2), namely

Gly-Cys-Arg-Asp

The trypsin digestion of the B₁, B₂ peptides yields the same structure except that trypsin splits between the arginine and aspartic acid residues. It is probable that the presence of a carboxamidomethyl group on the —SH group hinders the attack of pepsin to the right of the aspartic acid residue and that longer, more basic variants are produced than in the case of the pepsin digestion of the nonalkylated protein (2). In fact, some of this carboxamidomethylated tetrapeptide is present in small amounts in the pepsin digests of the alkylated protein. It contaminates the neutral region and might be assigned to peptide N in the kinetic analysis described below. Fortunately, it becomes more basic than peptide N at pH 3.5, and the two can be separated by electrophoresis at that pH, permitting a correction to be made to the relative proportion of radioactive label in the two peptide sequences. Such corrections have been made in the data used for the kinetic analyses described below.

Kinetics of Alkylation of Specific —SH Groups

In an attempt to correlate the enzymatic inactivation with the reaction of specific peptides with iodoacetamide, the kinetics of incorporation of radioactive reagent into each peptide was studied in comparison with the loss of catalytic activity. Samples were taken at various times from the reaction mixtures described above, treated with mercaptoethanol, and digested with pepsin, and the radioactive peptides were separated by paper electrophoresis at pH 6.5 and 3.5, as illustrated in Fig. 4. The area under the peak for each peptide was then measured. A direct plot of the areas so determined for the alkylation of phosphorylase a is shown in Fig. 5. In interpreting Fig. 5, it should be recalled that a total
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Fig. 5. Reaction of phosphorylase a peptides with iodoacetamide. Areas of peaks such as those depicted in Fig. 4 were measured with a planimeter and are expressed here in arbitrary units.

Fig. 6. Kinetics of incorporation of 14C-iodoacetamide into phosphorylase a peptides. Log a/a - x for each peptide was calculated as described in the text. The peptides marked C, D, and E did not appear in this experiment. Enzymatic activity was determined for the same mixture at the same time. A, alkylation of peptide A; O, alkylation of peptide N; 0, sum of alkylation of peptides A and N; X, activity loss.

of three —SH groups per monomer are reacting with iodoacetamide. It appears that the peptide B —SH groups reacts very rapidly compared to the other —SH groups. In this experiment, the total extent of its reaction took place within the 30 min before the first sample was taken. As suggested before, B1 and B2 appear to be variants of the same peptide and its alkylation has little effect on enzymatic activity. The pattern of labeling of peptide C varies from one experiment to the total but it remains an insignificant part of the total. Peptides N and A are being labeled progressively during the course of the reaction, suggesting that these two peptides are concerned with the inactivation. They are distinguishable and unique (already proven by the sequence determination) and they react at different rates with iodoacetamide. Peptides E and D, which are evident only during the later part of the reaction, may be variants of either peptide A or N, or both of them, since there is a similarity in the labeling pattern. These minor peaks do not appear in all experiments.

The areas shown in Fig. 5 and the areas from similar experiments with phosphorylase b were used to calculate reaction rates for the peptides. The total area of the peaks at each time was set equal to the total radioactivity (in disintegrations per min in a protein sample precipitated with trichloroacetic acid) incorporated at that time. From the area of each peptide as a percentage of the total area, the disintegrations per min incorporated into each peptide were determined. The extent of reaction of each peptide at each time could then be calculated by reference to the original specific radioactivity of the iodoacetamide. Representative pseudo-first order plots comparing the alkylation of the peptides with the loss of activity are shown in Fig. 6 for phosphorylase a and in Fig. 7 for phosphorylase b. The rate constants for these and other experiments, together with a rate calculated from Fig. 10 for peptide D, are summarized in Table II.

Fig. 7. Kinetics of incorporation of 14C-iodoacetamide into phosphorylase b peptides. Experimental procedure and calculations are specified in Fig. 6. The peptides marked C, D, and E in Fig. 4 did not appear in this experiment. X, loss of enzymatic activity; O, alkylation of peptide N; 0, alkylation of peptide A.

Table II

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Phosphorylase a</th>
<th>Phosphorylase b</th>
</tr>
</thead>
<tbody>
<tr>
<td>A + N</td>
<td>13.5 $\times 10^{-4}$ min$^{-1}$</td>
<td>2.9 $\times 10^{-4}$ min$^{-1}$</td>
</tr>
<tr>
<td>N</td>
<td>10.7 $\times 10^{-4}$ min$^{-1}$</td>
<td>9.9 $\times 10^{-4}$ min$^{-1}$</td>
</tr>
<tr>
<td>A</td>
<td>5.4 $\times 10^{-4}$ min$^{-1}$</td>
<td>6.1 $\times 10^{-4}$ min$^{-1}$</td>
</tr>
<tr>
<td>B1 and B2</td>
<td>3.1 $\times 10^{-4}$ min$^{-1}$</td>
<td>3.8 $\times 10^{-4}$ min$^{-1}$</td>
</tr>
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</table>

* Including variants D and E as described in text. D and E did not appear in the other experiments.

* Iodoacetamide, 1 mM, was used instead of 10 mM; the actual rate was corrected by multiplying by 10.
These kinetic studies suggest that the relationship to enzymatic activity of the two —SH groups per monomer in the sequences of peptides N and A depends on the form of the enzyme being studied. The rate constant for the alkylation of the —SH group of peptide N is similar to that for inactivation in the case of phosphorylase b, and we may conclude that the modification of only this single —SH group is sufficient to inactivate the enzyme. The kinetics of the alkylation suggest that the —SH group of peptide A becomes alkylated at the same time but that this is not primarily responsible for the activity loss. This interpretation agrees with the stoichiometry of the iodoacetamide incorporation, previously reported (1), which indicated that the activity loss was a linear function of the alkylation of two —SH groups.

In contrast, the results for phosphorylase a suggest that the sum of the reaction rates for peptides A and N is close to the over-all rate of inactivation. An analysis of the data by the method of Ray and Koshland (14, 15) suggests that the reaction of one or the other —SH group results in an inactivated form of the enzyme. Fig. 8 shows the stoichiometric relationship between the activity loss and the incorporation of radioactive iodoacetamide, and the results suggest that the reaction of either group tends to make the other unreactive. The earlier data for phosphorylase b are reproduced in Fig. 8 because this illustrates again the difference between the two forms of the enzyme.

The results with phosphorylase a, like those of phosphorylase b, suggest that the alkylation of only the —SH group of peptide N would be sufficient to inactivate the enzyme. Dr. O. Avramovic has confirmed this prediction by showing that, in the presence of 3 mM AMP, no alkylation of peptide A of phosphorylase a occurs and the cause of the inactivation must be assigned to the alkylation of peptide N.

**Peptide B**—Further evidence to support the conclusion that B1 and B2 (as denoted in Fig. 4) are variants of the same peptide rather than two unique peptides is shown in Fig. 9. The top line shows the total incorporation of 14C-iodoacetamide, calculated on the basis of three —SH groups per monomer of phosphorylase a, while the bottom line shows the reaction of the sum of B1 and B2 calculated as described in the previous section. The data for B1 and B2 do not show any significant change with time and, on averaging, indicate that 79.5% ± 1.8 of a single —SH group per monomer has reacted very rapidly. When the top line is extrapolated to zero time, it may be seen that 72% of all three groups are slow to react with iodoacetamide; that is, 28%, equivalent to 79.5% of one-third of the total groups, have reacted rapidly.

In another experiment on phosphorylase b the incorporation of 14C-iodoacetamide was studied at a concentration of 1 mM reagent so that the reaction would be slow enough to measure the rate of incorporation into peptide B. Table III compares the enzymatic activity with the amount of iodoacetamide incorporated per mole of dimer. It is evident that no enzymatic activity is lost while 1.6 moles of iodoacetamide are incorporated, equivalent to 80% of one —SH group per monomer. Electrophoresis of the pepsin digest at pH 6.5 indicated that nearly all of the label was in the B1 and B2 region (as shown in Fig. 4), while the small quantity (15%) appearing in the neutral region was shown to be the peptide Gly-Cys-Arg-Asp. Thus, under these conditions, no labeling of peptides N and A occurred. In view of our earlier results and conclusions, this result is consistent with there having been no loss of catalytic activity.

The reaction of only 80% of the B peptide is consistent with earlier results such as that shown in Fig. 9. There is previous

![Fig. 8. Effect of incorporation of 14C-iodoacetamide (IAM) on the enzymatic activity of glycogen phosphorylase.](image)

![Fig. 9. Total incorporation of 14C-iodoacetamide into phosphorylase a compared to the reaction of the B peptide.](image)

**Table III**

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Specific enzymatic activity</th>
<th>Iodoacetamide incorporated (mols/mole phosphorylase)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>56.7</td>
<td>0.10</td>
</tr>
<tr>
<td>5</td>
<td>61.6</td>
<td>0.84</td>
</tr>
<tr>
<td>20</td>
<td>68.0</td>
<td>1.32</td>
</tr>
<tr>
<td>40</td>
<td>63.7</td>
<td>1.61</td>
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<tr>
<td>60</td>
<td>67.0</td>
<td>1.60</td>
</tr>
<tr>
<td>80</td>
<td>63.3</td>
<td>1.50</td>
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<tr>
<td>100</td>
<td>63.3</td>
<td>1.67</td>
</tr>
<tr>
<td>120</td>
<td>60.0</td>
<td>1.38</td>
</tr>
</tbody>
</table>

2 O. Avramovic, unpublished observations.
The possible role of this highly reactive SH group in modulating the regulation of activity of this complicated allosteric enzyme remains in doubt, but the formation of similar mixed disulfides with fructose diphosphatase has been shown to affect the activity of that enzyme (20). In addition, it should be noted that Kastenschmidt, Kastenschmidt, and Heimreich (21) have recently shown that reaction of phosphorylase b with DTNB results in the production of three species of enzyme, which are separable by sucrose density gradient centrifugation. One of these species is a fully active dimer with up to three of its —SH groups blocked but which has lost part of its allosteric response to AMP in that it no longer displays homotropic cooperativity to that ligand. We find, however, that phosphorylase b prepared with its B peptide fully alkylated retains all of its normal allosteric responses to AMP, ATP, and glucose-1-P. The only effect appears to be a slight increase of activity (as in Fig. 10) and a slight decrease in the apparent $K_m$ values for AMP and glucose-1-P. The difference between the results of Heimreich's group and our own may be due to the considerable difference in reagents, or it may be that a second peptide is being attacked by the DTNB. It is not impossible that a reagent other than iodoacetamide might react with the —SH group of peptide A without touching that of peptide N and thereby causing inactivation. Thus, the —SH of peptide A may be a candidate for being involved in the one contact region per monomer which Heimreich suggests may be responsible for the allosteric properties of phosphorylase b (21).

For phosphorylase b, both the loss of activity and the dissociation into monomers appear to be well correlated with the alkylation of the —SH group of peptide N. We can only speculate at this point about the possible role of peptide A. The situation with respect to phosphorylase a is at once simpler and more complex. Because AMP prevents peptide A from being alkylated at all, we are able to assign the loss of activity in the presence of AMP entirely to the alkylation of peptide N. Nevertheless, an analysis of the kinetics by the method of Ray and Koshland (14, 15), as discussed earlier, suggests that the alkylation of either of peptide A or N results in an inactive enzyme. It should be noted, however, that this analysis does not take into account the possibility of cooperative effects in an oligomeric protein, and certain discrepancies in our data suggest this possibility. For example, Fig. 8 indicates that, at a certain region of the titration of activity by iodoacetamide, activity is being lost faster than the alkylation of even a single —SH group per monomer. Furthermore, Fig. 3a shows that the extent of dissociation of phosphorylase a is less than the extent of inactivation. It may be, then, that the alteration of only one peptide N in each oligomer may result in an inactive molecule of phosphorylase a, the conformational change in one monomer being transmitted to the other.

The sequence of events which lead to inactivation and dissociation is of considerable interest. Since the alkylation of peptide N —SH is several hundred times slower than that of peptide B or model compounds, it must be protected in some manner. It was suggested by Cecil and Thomas (22) and confirmed by Perutz (23) that certain —SH groups in hemoglobin are buried in hydrophobic regions. If this situation exists in phosphorylase, then certain protein conformations may exist, to a small degree and in equilibrium with the more stable native conformation, in which the —SH group of peptide N becomes available for alkylation. Once a reagent has become attached to peptide N, the
original native conformation cannot be regained, and the protein undergoes further conformational states which lead first to loss of enzymatic activity and second to dissociation. Already in 1956, Madsen, Cori, and Gurd had suggested a similar sequence for the inhibition by CMB because the reaction of this reagent with the —SH groups, measured by an optical method, was faster than the inactivation, which in turn was very much faster than the dissociation, the latter having been measured by light scattering (12, 24, 25). At that time, with CMB, we could not distinguish readily between various types of —SH groups or associate the events with a single unique cysteine residue in each monomer.

Gerhart and Schachman (26), in discussing a similar situation, have suggested that the enzyme aspartic transcarbamylase exists to a very small extent in a dissociated form in which the —SH groups are exposed and react readily with CMB. The earlier work cited above indicates that a more complicated sequence exists for phosphorylase.

While this work has been in progress, a number of other papers have appeared in which studies on the —SH groups of phosphorylase are reported. In general, the results agree with ours although, as we have found, it is sometimes difficult to state decisively the exact number of —SH groups involved in each effect until the modified protein can be characterized chemically. Some of these papers were discussed in Reference 1. Jokay, Damjanovich, and Toth (18), Damjanovich and Kleppe (27), and Damjanovich, Sanner, and Phil (28) have presented evidence that there is an interrelationship between AMP binding and certain —SH groups, and we would now suggest this —SH group to be that of peptide A, at least in the case of phosphorylase a. Gold (29) finds that dinitrophenylating agents react rapidly with four —SH groups per phosphorylase b dimer with little effect on catalytic activity or molecular weight but an increase in the apparent $K_m$ values for AMP and glucose-1-P. His results, particularly the finding of four reactive —SH groups instead of two, are difficult to correlate precisely with those reported here but the isolation and identification of the peptides involved will no doubt clear up the matter and may illuminate further the role of each —SH group. In particular, the work of Johnson, Philip, and Graves (30) with the dinitrophenylated enzyme suggests that changes in the enzymatic activity might result from something more complicated than merely chemical modification of certain functional groups. If this is so, then we must expect considerably different characteristics in phosphorylases which have had their —SH groups modified by the different reagents used.

Acknowledgments—We are indebted to Dr. Olga Avramovic for the data in Figs. 3a and 6, and for her valuable collaboration. Mrs. Shirley Shechosky and Miss Christine Naylor provided skilled technical assistance while Mr. Alan Davidson performed the amino acid analyses and Mr. Morris Aarbo the ultra-centrifugal analyses. We also thank Dr. E. Holmreich for permission to quote a manuscript in press.

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