The Inactivation of the Acyl Phosphatase Activity Catalyzed by the Sulfenic Acid Form of Glyceraldehyde 3-Phosphate Dehydrogenase by Dimedone and Olefins*

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SUMMARY

Treatment of the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12) with a 2-fold molar excess of dimedone over the concentration of enzyme subunit completely inactivates the acyl phosphatase reaction catalyzed by the oxidized enzyme. The dehydrogenase activity catalyzed by the reduced form of the enzyme is not inactivated when the dimedone-inactivated enzyme is treated with dithiothreitol. When the acyl phosphatase is inactivated by $[^{14}C]$dimedone $\sim 1 \mu g$ atom of $^{14}C$ is incorporated per $\mu$eq of oxidized enzyme subunit which is not removed by gel filtration on Sephadex G-25. A $^{14}C$-labeled peptide from a tryptic digest of the acyl phosphatase which was inactivated with $[^{14}C]$dimedone has been isolated in pure form and has been subjected to sequence analysis. This analysis has shown that $[^{14}C]$dimedone forms a thioether derivative of Cys-149 by reacting with the sulfenic acid at the active site of the acyl phosphatase. The dehydrogenase activity is unaffected by $[^{14}C]$dimedone when reduced glyceraldehyde 3-phosphate dehydrogenase is treated with $[^{14}C]$dimedone and $^{14}C$ radioactivity is not incorporated into the reduced protein.

The acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase isolated from pig muscle is also inactivated by 25 mM 3-cyclohexene-1-carboxylate at pH 5.1 in the presence of 0.1 M (NH4)$_2$SO$_4$ and other salts. Treatment of the inactivated acyl phosphatase with dithiorethitol does not reactivate the dehydrogenase activity catalyzed by native glyceraldehyde 3-phosphate dehydrogenase when added to it directly. Amino acid sequence analysis of the tryptic peptide which contains Cys-149 after the acyl phosphatase was inactivated with tetrahydrophthalimide suggests that an adduct between the sulfenic acid at the active site of the acyl phosphatase and tetrahydrophthalimide is formed during the inactivation.

The oxidation of the sulfhydryl group at the active site of glyceraldehyde 3-phosphate dehydrogenase to a sulfenic acid converts the dehydrogenase to an acyl phosphatase (1–3). Evidence has been presented which suggests that the sulfenic acid residue participates directly in the hydrolytic activity as described by Equations 1 and 2.

$$\text{ESON} + R-C-O-PO_{3}H^+ \rightarrow \text{ESO}^+ + R-C-O-PO_{3}^{-} + H^+$$

$$\text{ESO}^{-} + R-C-R + H_2O \rightarrow \text{ESOH} + ROH + H^+$$

A number of nucleophiles which are classical carbonyl reagents inactivate the acyl phosphatase activity of the enzyme by forming sulfenyl derivatives of cysteine residue 149. Among these are cyanide, bisulfite, semicarbazide, and hydrazines (2, 4, 5).

Sulfenyl halides are known to react with active methylene compounds to form the corresponding thioethers (6). It was therefore of interest to see if a protein sulfenic acid would also react with active methylene compounds. Experimental evidence is presented which supports such a reaction of $[^{14}C]$dimedone with the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase.

Sulfenyl halides and other sulfenyl derivatives such as sulfenyl carboxylates are known to add across olefinic bonds by a reaction similar to the addition of Br$_2$ across olefinic bonds. The addition of sulfenyl compounds to olefins proceeds with the formation of an episulfonium intermediate as shown by Equation 3.

$$RSX + \longrightarrow [RS_2X^+] \rightarrow R^-S$$

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The product of the reaction is invariably the trans-β-substituted thioether where X− is a component of the solvent react with the episulfonium intermediate (7, 8).

We wish to report that water-soluble olefins also irreversibly inactivate the acyl phosphatase reaction catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase. In addition evidence is presented that tetrahydrophthalimide reacts with the sulfenic acid form of the enzyme to form a covalent derivative of Cys-149.

**EXPERIMENTAL PROCEDURE**

**Materials**

Glyceraldehyde 3-phosphate dehydrogenase was prepared from pig muscle as described by Elödi and Szörényi (9) with a modification described previously (10). The enzyme was oxidized to the sulfenic acid form in the following manner. Crystals of the dehydrogenase were collected by centrifugation and were dissolved in 20 mM Veronal, pH 7.6, to give a final protein concentration of 20 to 25 mg per ml. A neutralized solution of o-iodosobenzene was added to the dissolved enzyme in the presence of 10 mM NaOH to a final concentration of 20 mM. Oxidation was carried out for 1 hour at 0°C at which time excess reagents were removed by gel filtration on a column (2.0 X 30 cm) of Sephadex G-25 (medium) which was equilibrated and eluted with 20 mM Veronal buffer, pH 7.6. The concentrated protein fractions were combined and maintained at 0°C until they were used in the various experiments. All experiments were performed within 8 hours after the isolation of the oxidized enzyme.

(14) Dimedone was generously provided by Dr. David Sigman of the University of California, Los Angeles, School of Medicine. Spectrophotometric grade nitromethane was purchased from the University of California, Los Angeles, School of Medicine. Oxidized enzyme.

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The optical density of the 5.5-ml collected fractions was determined at 280 nm ( ), 100-μl samples of these fractions were monitored for 14C radioactivity ( ).

Fig. 1 (left). Fractionation on Sephadex G-25 of the tryptic digest of [14C]Dimedone-inactivated carboxymethylated oxidized enzyme. The tryptic digest was prepared and was fractionated on Sephadex G-25 as described under “Experimental Procedure.” The optical density of the 5.5-ml collected fractions was determined at 280 nm ( ), 100-μl samples of these fractions were monitored for 14C radioactivity ( ).

Fig. 2 (center). Fractionation on Sephadex G-50 of the radioactive fraction obtained from Sephadex G-25 gel filtration. The lyophilized radioactive Fractions 43 to 53 illustrated in Fig. 1 were gel-filtered on Sephadex G-50 as described under “Experimental Procedure.” Five-milliliter fractions were collected ( ), optical density at 280 nm ( ), 14C counts per min for 100-μl sample ( ).

Fig. 3 (right). DEAE-Sephadex A-25 column chromatography of the radioactive fraction from Sephadex G-50 gel filtration. The lyophilized radioactive fractions 45 to 68 illustrated in Fig. 2 were applied to a DEAE-Sephadex A-25 column and chromatographed as described under “Experimental Procedure” ( ). Fifty-microliter samples of the collected fractions were assayed at 750 nm by the method of Lowry et al. (14).
The purity of the [14C]dimedone-labeled active site tryptic peptide was monitored by high voltage electrophoresis on Whatman No. 3MM paper using 1% (NH₄)₂CO₃, pH 8.9, as electrolyte.

**Amino Acid Analysis**

Peptide samples for amino acid analysis were prepared as follows. About 15 to 25 nmoles of the peptide in 1.0 ml of 0.05 M NH₄OH-0.1% thiodiglycol were extracted with 1.0 ml of benzene three times, to remove the thiodiglycol. The aqueous peptide solution was then lyophilized and then hydrolyzed with 300 μl of constant boiling HCl in vacuum-sealed tubes at 110°C for 24 or 48 hours. When thiodiglycol was not present about 15 to 25 nmoles of peptide were lyophilized and were hydrolyzed with 300 μl of constant boiling HCl in vacuum-sealed tubes at 110°C for 24 or 48 hours. Analyses were conducted with the use of a Beckman model 119 automatic amino acid analyzer (15).

**RESULTS**

**Inactivation of Acyl Phosphatase Activity by Dimedone and Nitromethane**—Table I shows that the acyl phosphatase reaction catalyzed by oxidized glyceraldehyde 3-phosphate dehydrogenase is inactivated by fairly low concentrations of dimedone. For instance, when a stoichiometric concentration of dimedone is added to the oxidized enzyme at room temperature, 55% of the acyl phosphatase is lost in 30 min. Fig. 4 shows the time dependence of the inactivation of the acyl phosphatase activity when the oxidized enzyme is treated with a 2-fold molar excess of dimedone over the concentration of enzyme subunit. The oxidized enzyme is completely inactivated by dimedone within 1 hour at room temperature while the oxidized enzyme in an untreated control lost approximately 15% of its activity during a similar incubation. Treatment of the control reaction mixture at the end of 1-hour incubation with 0.01 M dithiothreitol for 30 min at room temperature led to the reactivation of 90% of the dehydrogenase activity catalyzed by reduced glyceraldehyde 3-phosphate dehydrogenase. No dehydrogenase activity was recovered when the reaction mixture which contained dimedone was treated with dithiothreitol. This suggests that treatment of the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase with dimedone leads to the covalent modification of Cys-149. When reduced glyceraldehyde 3-phosphate dehydrogenase is treated with 1 mM dimedone, the dehydrogenase activity is not affected. This also suggests that dimedone reacts with the sulfenic acid at the active site of the acyl phosphatase.

Nitromethane, another active methylene compound with a much higher pKₐ than dimedone, inactivates the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase but much less effectively than dimedone. For instance a 2-fold molar excess of dimedone over the concentration of enzyme subunit completely inactivates the acyl phosphatase after an hour, while a 200-fold molar excess of nitromethane results in only 30% inactivation of the acyl phosphatase under the same conditions.

**TABLE I**

| Dimedone concentration | Incubation time | Per cent inactivation |
|------------------------|----------------|----------------------|
| mM                     | min            |                      |
| 0.10                   | 30             | 55                   |
| 0.20                   | 30             | 70                   |
| 0.50                   | 30             | 100                  |
| 1.0                    | 30             | 100                  |
In Experiment 1 the oxidized enzyme was incubated with \[^{14}C\]dimedone in 20 mM Veronal buffer, pH 7.6, in a total volume of 5.22 ml for 30 min at room temperature at which time the oxidized enzyme was completely inactivated. The reaction mixture was then gel-filtered on a column (2.0 X 50 cm) of Sephadex G-25, equilibrated, and eluted with the same buffer. The radioactivity protein fractions were combined and 100-μl samples were counted for \[^{14}C\]dimedone incorporation. Experiments 2 and 3 were performed similarly. The total volume of the reaction mixture in Experiment 2 was 9.61 ml and the reaction mixture was incubated for 60 min at room temperature. The total volume of the reaction mixture in Experiment 3 was 7.20 ml and it was incubated for 40 min at room temperature. Experiments 4 and 5 were conducted under identical reaction conditions. In these experiments, the enzyme and \[^{14}C\]dimedone, in a total volume of 2.0 ml Veronal buffer, pH 7.6, were incubated for 20 min at room temperature. The reaction mixture was then gel-filtered on a column (2.0 X 50 cm) of Sephadex G-25 equilibrated and eluted with the same buffer. A 50-μl sample from the combined radioactive protein fractions was counted for \[^{14}C\]dimedone incorporation. At the end of the 20-min incubation period, the acyl phosphatase activity of the oxidized enzyme was completely destroyed but the dehydrogenase activity of the reduced enzyme remained unchanged.

**Table II**

Incorporation of \[^{14}C\]dimedone into oxidized reduced glyceraldehyde 3-phosphate dehydrogenase

| Experiment | Enzyme | \[^{14}C\]Dimedone | \[^{14}C\]Dimedone incorporated | Amount of \[^{14}C\] per \(\mu g\) of enzyme |
|------------|--------|---------------------|-----------------------------|----------------------------------|
| 1          | 1.0 ESOH | 10.05              | 6.00 x 10^4                 | 4.80 x 10^4 0.78                 |
| 2          | 4.0 ESOH | 8.00                | 1.25 x 10^5                 | 5.10 x 10^4 1.02                 |
| 3          | 2.0 ESOH | 4.40                | 2.75 x 10^4                 | 2.55 x 10^4 0.46^               |
| 4          | 0.10 ESOH | 1.13            | 5.51 x 10^4                 | 4.99 x 10^4 0.89                 |
| 5          | 0.10 ESH  | 1.13               | 5.32 x 10^4                 | 2.0 x 10^3 1.00^                 |

*This value may be low due to a counting error. Amino acid analysis of a radioactive peptide purified from a tryptic digest of the enzyme labeled in this experiment suggests that approximately 0.7 g atom of \[^{14}C\] was incorporated per \(\mu g\) of enzyme.

A radioautograph of the subtilisin digest of the \[^{14}C\] labeled tryptic peptide isolated in the presence of thioglycol. The \[^{14}C\]dimedone-labeled tryptic peptide was digested with subtilisin as described in the text. The freeze-dried digest was dissolved in 0.05 M NH₄OH which contained 0.1% triethanolamine and was applied to Whatman No. 3MM paper and was subjected to electrophoresis for 50 min at pH 3.5 at 3 kv.

**Table III**

Amino acid compositions of \[^{14}C\] labeled peptides

| Amino Acids     | Active subtilisin peptide[a] | \[^{14}C\] Dimedone (24 hr hydrol.) | \[^{14}C\] Dimedone-labeled S₄ (24 hr hydrol.) | \[^{14}C\] Dimedone-carboxymethylated tryptic peptide (24 hr hydrol.) |
|-----------------|------------------------------|-----------------------------------|---------------------------------------------|-----------------------------------------------------------------|
| Lysine          | 1                            | 1.00                              | 1.00                                        | 1.00                                                            |
| Cysteine-SO₄⁻   | 2                            | 0.28                              | 0.21                                        | 0.73                                                            |
| Carboxymethylleucine | 3                        | 0.75                              | 0.81                                        | 0.81                                                            |
| Aspartic acid   | 4                            | 2.15                              | 2.08                                        | 2.08                                                            |
| Threonine       | 2                            | 1.90                              | 2.13                                        | 1.96                                                            |
| Serine          | 2                            | 2.03                              | 1.04                                        | 1.92                                                            |
| Proline         | 2                            | 0.81                              | 0.93                                        | 0.93                                                            |
| Alanine         | 3                            | 3.33                              | 1.00                                        | 3.18                                                            |
| Valine          | 1                            | 0.87                              | 0.48                                        | 0.48                                                            |
| ISOleucine      | 1                            | 0.87                              | 0.56                                        | 0.56                                                            |
| Leucine         | 2                            | 1.84                              | 2.02                                        | 2.02                                                            |
| Half-cystine    | 2                            |                                   |                                             |                                                                 |

[a] Based on the sequence published by Harris and Perham (18).
[b] Calculated on the basis of 1.00 residue of lysine.
[c] Calculated on the basis of 1.00 residue of alanine.

**Fig. 5.** A radioautogram of the subtilisin digest of the \[^{14}C\] labeled tryptic peptide isolated in the presence of thioglycol. The \[^{14}C\]dimedone-labeled tryptic peptide was digested with subtilisin as described in the text. The freeze-dried digest was dissolved in 0.05 M NH₄OH which contained 0.1% triethanolamine and was applied to Whatman No. 3MM paper and was subjected to electrophoresis for 50 min at pH 3.5 at 3 kv.
Sequence of peptide S4

The experimental procedures for the dansyl-Edman sequence determination are described and documented in the text.

| Edman step | Peptide sequence          | Total | Dansyl-NH2-terminal determination | No. of dansyl steps |
|------------|---------------------------|-------|-----------------------------------|---------------------|
| 0          | Ala-(Ser,Cys,Thr,Thr)     | 33,300| Dns-Ala                           | 3                   |
| 1          | Ala-Ser-(Cys,Thr,Thr)     | 8,000 | Dns-Ser                           | 3                   |
| 2          | Ala-Ser-(?,Thr,Thr)       | 130   | Dns-Thr                           | 2                   |

The abbreviation used is: dansyl, DNS, 5-dimethylamino-naphthalene-1-sulfonyl.

The acid composition of the peptide S4 purified in this manner is shown in Table III.

Based on specific radioactivity, 30 nmols of peptide S4 were subjected to sequence analysis by the dansyl-Edman procedure of Gray (19) as shown in Table IV. The dansyl-amino acids were identified on polyamide thin layer sheets described by Woods and Wang (20) with the solvent systems described by Hartley (21). The NH2-terminal amino acid was identified as dansyl-alanine and after the first Edman step the new NH2-terminal amino acid was identified as dansyl-serine. To prove unequivocally that alanine was the NH2-terminal amino acid and serine was the new NH2-terminal amino acid after the first Edman step, three separate dansyl steps were made in each case. This consumed a considerable portion of the radioactive peptide. Before the second Edman step, 8000 cpm of 14C remained. No dansyl-amino acid, including dansyl-cysteic acid was identified as dansyl-serine. The third Edman step removed 90% of the remaining radioactivity, indicating that the 3rd residue in the sequence of peptide S4 was labeled with [14C].

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To prove unequivocally that alanine was the NHz-terminal amino acid after the first Edman step, three separate dansyl steps were made in each case. This consumed a considerable portion of the radioactive peptide. Before the second Edman step, 8000 cpm of 14C remained. No dansyl-amino acid, including dansyl-cysteic acid was identified as dansyl-serine. The third Edman step removed 90% of the remaining radioactivity, indicating that the 3rd residue in the sequence of peptide S4 was labeled with [14C].

Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-

Cys-149 is marked with an asterisk and was shown to be labeled with [14C]dimedone by this sequence analysis.

Removal of [14C]Dimedone from Peptide S4 by Performic Acid Oxidation—A sample of peptide S4 which contained 3.1 \times 10^4 cpm of 14C was applied as a 1.5-cm band on a strip of Whatman No. 3MM paper which was exposed to performic acid vapor in a vacuum desiccator for 2 hours at room temperature. The paper strip containing the performic acid-oxidized sample of peptide S4 was sewn onto a full sheet of Whatman No. 3MM paper and subjected to sequence analysis by the dansyl-Edman procedure of Gray (19) as shown in Table IV. The dansyl-amino acids were identified on polyamide thin layer sheets described by Woods and Wang (20) with the solvent systems described by Hartley (21). The NH2-terminal amino acid was identified as dansyl-alanine and after the first Edman step the new NH2-terminal amino acid was identified as dansyl-serine. To prove unequivocally that alanine was the NH2-terminal amino acid and serine was the new NH2-terminal amino acid after the first Edman step, three separate dansyl steps were made in each case. This consumed a considerable portion of the radioactive peptide. Before the second Edman step, 8000 cpm of 14C remained. No dansyl-amino acid, including dansyl-cysteic acid was identified as dansyl-serine. The third Edman step removed 90% of the remaining radioactivity, indicating that the 3rd residue in the sequence of peptide S4 was labeled with [14C].

When the ionogram was stained with the ninhydrin-cadmium reagent, ninhydrin-positive material appeared under the radioactive spot of the radioautogram, under the untreated peptide S4, and a ninhydrin-positive spot was detected where the performic acid-treated peptide S4 was applied which was more acidic than the untreated peptide S4. These experiments also suggest that [14C]dimedone formed a covalent derivative of Cys-149.

Inactivation of Acyl Phosphatase Activity Catalyzed by Oxidized Glyceraldehyde 3-Phosphate Dehydrogenase with 3-Cyclohexene-1-Carboxylate, Dihydropyran, and Tetrahydrophthalimid

Table V shows that incubation of oxidized glyceraldehyde 3-phosphate dehydrogenase (3.6 mg per ml) with 25 mM 3-cyclohexene-1-carboxylate at pH 5.1 in the presence of 0.1 mM (NH4)2SO4 leads to 94% inactivation of the acyl phosphatase within 15 min at room temperature when this activity is assayed at pH 7.6 (1). However, this olefin does not inactivate the acyl phosphatase at pH 5.1 in the absence of (NH4)2SO4 and in fact it appears to stabilize the oxidized enzyme in the absence of the salt. The addition of 0.1 mM (NH4)2SO4, to the oxidized enzyme in the absence of 3-cyclohexene-1-carboxylate also stabilized the oxidized enzyme slightly at pH 5.1 when compared to the untreated control as shown in Table V. The reaction mixtures which were incubated under the various conditions shown in Table V were prepared in a manner to ensure that each had a final pH of 5.1 as determined by a micro glass electrode. Therefore, differences in pH are not responsible for the results shown in Table V. At pH values greater than 5.1, the acyl phosphatase was progressively less sensitive to inactivation by 3-cyclohexene-1-carboxylate in the presence of 0.1 mM (NH4)2SO4. At pH 6.0 and above 25 mM 3-cyclohexene-1-carboxylate does not inactivate the acyl phosphatase in the presence of added (NH4)2SO4.

Table V also shows that the inactivation of the acyl phos-
The reaction mixtures contained 0.05 μeq of oxidized enzyme; and where indicated 12.5 μmoles of 3-cyclohexene-1-carboxylate and 50 μmoles of (NH₄)₂SO₄ in a total volume of 0.50 ml of 0.1 M acetate buffer. The final pH of each of the reaction mixtures was 5.1. The reaction mixtures were incubated for 15 min at room temperature and at the times indicated 0.10-ml samples were assayed for acyl phosphatase activity by the method of Ehring and Colowick (1). Simultaneously, 0.05-ml samples were diluted to 1.0 ml with 0.05 mM pyrophosphate, pH 8.5, which contained 0.01 mM dithiothreitol. Samples of 0.05 ml each of the dilutions in dithiothreitol were assayed for dehydrogenase activity after incubating for 60 min at room temperature.

When cyclohexane monocarboxylate is incubated with the oxidized enzyme, 0.10 μeq, at pH 5.1, in the presence and absence of added (NH₄)₂SO₄ at pH 5.1 does not inactivate the dehydrogenase activity when assayed at pH 8.5. These observations suggest that the sulfenic acid at the active site of the acyl phosphatase might add to the olefinic bond of 3-cyclohexene-1-carboxylate as described by Equation 3 at pH 5.1 in the presence of 0.1 M (NH₄)₂SO₄.

To investigate the curious requirement of (NH₄)₂SO₄, for the inactivation of the acyl phosphatase by 3-cyclohexene-1-carboxylate further, the oxidized enzyme (3.5 mg per ml) was incubated with varying concentrations of different salts for 15 min at room temperature in the presence of 25 mM 3-cyclohexene-1-carboxylate at pH 5.0 at which time samples of the reaction mixtures were assayed. Of the salts tested over the concentration range of 0 to 200 mM, (NH₄)₂SO₄ was the most effective in promoting the inactivation by this olefin. For instance under these reaction conditions, 70% inactivation of the acyl phosphatase was observed in the presence of 0.15 M (NH₄)₂SO₄, while the addition of 0.15 M NaSO₄, 0.15 M NH₄Cl, and 0.15 M NaCl resulted in 88, 80, and 87% inactivation of the acyl phosphatase, respectively.

Fig. 7 shows that 5 mM dihydropyran completely inactivates the acyl phosphatase reaction catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase within 45 min at room temperature at pH 7.5. Addition of dithiothreitol to the inactivated acyl phosphatase did not reactivate the dehydrogenase activity, indicating that a covalent modification of the enzyme had occurred. Dihydropyran has been shown to react with sulfenyl compounds to form substituted thiocarbamates (8). When the reduced form of glyceraldehyde 3-phosphate dehydrogenase is treated with dihydropyran under the same conditions, the dehydrogenase activity is not inactivated.

Table V

| Incubation mixture | Acyl phosphatase after 15 min at pH 5.1 | Dehydrogenase activity recovered by dithiothreitol |
|--------------------|------------------------------------------|-----------------------------------------------|
| ESOH               | %                                        | %                                            |
| ESOH + 0.1 M (NH₄)₂SO₄ | 92.0                                    | 91.6                                         |
| ESOH + 25 mM 3-cyclohexene-1-COO⁻ | 100                                     | 100                                           |
| ESOH + 0.1 M (NH₄)₂SO₄ + 25 mM 3-cyclohexene-1-COO⁻ | 6.1                                    | 5.0                                           |

Fig. 8. The inactivation of the acyl phosphatase activity catalyzed by oxidized glyceraldehyde 3-phosphate dehydrogenase by 5 mM dihydropyran. The reaction mixtures contained in 1.0 ml of 20 mM Veronal, pH 7.6, the oxidized enzyme, 0.10 μeq, and 5.0 mM dihydropyran. At the time intervals indicated, 0.10-ml samples were withdrawn from the reaction mixtures which were incubated at room temperature and assayed for acyl phosphatase activity by the method of Ehring and Colowick (1). ●, the reaction mixture which contained dihydropyran; ○, the reaction mixture with no additions.
activates the acyl phosphatase activity catalyzed by the sulfinic acid form of glyceraldehyde 3-phosphate dehydrogenase at pH 6.0 at room temperature within an hour. The addition of acetyl phosphate to a reaction mixture slightly enhanced the rate of inactivation of the acyl phosphatase by tetrahydrophthalimide. The addition of dithiothreitol to the inactivated acyl phosphatase did not lead to the recovery of the dehydrogenase activity, again indicating that a covalent modification of the enzyme had occurred. Tetrahydrophthalimide does not inactivate the dehydrogenase reaction catalyzed by reduced glyceraldehyde 3-phosphate dehydrogenase when added to it directly.

Isolation and Sequence Characterization of Tryptic Peptide Which Contains Cys 149 after Inactivation of Acyl Phosphatase Activity by Tetrahydrophthalimide—The amino acid sequence of pig muscle glyceraldehyde 3-phosphate dehydrogenase has been determined by Harris and Perham (18). Each of the identical subunits contains 3 cysteine residues in addition to Cys-149, the catalytically active sulfhydryl group. One of these, Cys-153, is 4 residues removed from Cys-149, and in tryptic digests of the enzyme, resides on the same peptide as Cys-149. Advantage was taken of this convenient feature to isolate specifically this tryptic peptide after the acyl phosphatase activity catalyzed by the sulfinic acid form of glyceraldehyde 3-phosphate dehydrogenase was inactivated irreversibly with tetrahydrophthalimide. The experimental procedure for this isolation is described below.

Oxidized glyceraldehyde 3-phosphate dehydrogenase (170 pg or 4.7 pmoles of enzyme subunit) was incubated with 33 mM tetrahydrophthalimide for 150 min in 14.8 ml of 0.1 M sodium succinate, pH 6.0, at which time 93% of the acyl phosphatase activity was inactivated. The pH of the reaction mixture was then adjusted to 7.8 by the slow addition of 1 M Tris base. Then 75 μmoles of [1-14C]iodoacetate with a specific radioactivity of 2.2 × 10⁶ cpm per μmole and solid urea to a final concentration of 8 M were added. The reaction mixture was warmed to room temperature and was incubated for 2 hours to carboxymethylate the available sulfhydryl groups with [1-14C]iodoacetate. Urea and excess reagents were then removed from the protein by exhaustive dialysis against 1 mM HCl. Trypsin, 1.7 mg, and solid urea to a final concentration of 0.5% were then added to the dialyzed protein. The resulting solution was then stirred slowly at 37° for 6 hours at which time the tryptic digest was freeze-dried. The freeze-dried digest contained 13.5 μg atom of 14C. If each of the four sulfhydryl groups per subunit had been alkylated with [1-14C]iodoacetate, it would have contained 19.8 μg atoms of 14C.

The radioactive peptides in the tryptic digest were partially purified by successive gel filtration steps on columns (3.0 × 100 cm) of Sephadex G-25 and Sephadex G-50, using 0.05 M NH₄OH as solvent. All of the radioactivity after the Sephadex G-25 step appeared in the void volume. The Sephadex G-50 gel filtration step resolved the radioactive material into two peaks. Peak SI which appeared near the void volume contained 4.5 μg atoms of 14C and contains the [1-14C]carboxymethyl derivative of Cys-281 which resides on a tryptic peptide which contains residues 269 to 306 (18).

Peak SII, the radioactive material which was retained on the Sephadex G-50 column was freeze-dried and contained 8.6 μg atoms of 14C. It was subjected to anion exchange chromatography with a pH gradient as described in the legend of Fig. 9. The elution profile of the DEAE-Sephadex fractionation of the peptides in Peak SII is shown in Fig. 9. The radioactive peaks labeled I, II, and III were pooled separately, freeze-dried, and desalted on a column (3.0 × 100 cm) of Sephadex G-25, using 0.05 M NH₄OH as solvent. After desalting, Peak I contained 2.2 μg atoms of 14C; Peak II contained 3.5 μg atoms of 14C; and Peak III contained 0.8 g atom of 14C.

Each of the radioactive peptides in Peaks I, II, and III was then purified to homogeneity by successive high voltage electrophoresis steps at pH 6.5 and 8.9. After elution from paper, samples of each of the radioactive peptides were subjected to amino acid analysis. These analyses revealed that the radioactive peptide purified from Peak I contained the [14C]carboxymethyl derivative of Cys-244 which resides on a tryptic peptide which contains residues 232 to 245 (18) and that the radioactive peptide purified from Peak III contained the [14C]carboxymethyl-cysteine sulfoxide derivative of the same peptide. It has been shown that the atmospheric oxidation of carboxymethylcysteine in peptides to the sulfoxide changes the ion exchange behavior of the peptides (16).

The amino acid composition of the [14C]carboxymethylated peptide purified from the DEAE-Sephadex Peak I, which was surprisingly acidic on electrophoresis at pH 6.5, is shown in Table III. It is clear that this peptide is a derivative of the tryptic peptide of pig glyceraldehyde 3-phosphate dehydrogenase which contains Cys-149. The amino acid analysis of this peptide showed that it contained approximately 1 residue of carboxymethylcysteine and approximately 1 residue of cysteic acid and no new ninhydrin-positive peak on the chromatogram that might be derived from a tetrahydrophthalimide derivative of Cys-149. Since cysteic acid appears near the void volume of the chromatographic system employed, the possibility that the apparent cysteic acid peak might represent the β-hydroxythioketone derivative of Cys-149 with two free ε-carboxyl groups as shown by Structure I was considered. To investigate this possibility, 0.6 μmole of the peptide (40% of the 1.5 μmoles isolated) were hydrolyzed with 6 M HCl at 110° in an evacuated sealed tube for 30 hours. The freeze-dried hydrolysate was then applied to a "
of the [\textsuperscript{14}C]carboxymethylated tryptic peptide containing a derivative of Cys-149 after inactivation of acyl phosphatase with tetrahydrophthalimide. The preparation of the radioautograph which superimposes a guide strip stained with ninhydrin is described in the text.

The material which migrated with cysteic acid at pH 1.9 had the same electrophoretic mobility as authentic cysteic acid when subjected to electrophoresis at pH 3.5, 6.5, and 8.9. It also had the same \( R_{f} \) as authentic cysteic acid when subjected to paper chromatography using butanol-pyridine-acetic acid-water (150:100:30:120) as solvent. Therefore, we conclude that the acid hydrolysis at 110\(^{\circ}\) of the tryptic peptide which contains Cys-149 which was isolated after inactivating the acyl phosphatase activity with tetrahydrophthalimide, and then carboxymethylating with [\textsuperscript{1-}\textsuperscript{14}C]iodoacetate gives rise to 1 residue of [\textsuperscript{14}C]carboxymethylcysteine and 1 residue of cysteic acid.

The cysteic acid identified in the acid hydrolysate described above may have been formed directly when the acyl phosphatase was inactivated with tetrahydrophthalimide or it may have been formed during acid hydrolysis of an adduct of Cys-149 in the tryptic isolated peptide by 6 M HCl at 110\(^{\circ}\). To distinguish between these possibilities, the remainder of the radioactive peptide, 0.9 \( \mu \)g, was digested with 50 \( \mu \)g of pronase for 18 hours at 37\(^{\circ}\) in 0.5 ml of 0.5% NH\(_{4}\)HCO\(_{3}\). The pronase digest was subjected to paper electrophoresis at pH 6.5 for 50 min at pH 6.5 at 3 kv. The ionogram was radioautographed and a guide strip was stained with the ninhydrin-cadmium reagent (17). Inspection revealed, in addition to a high concentration of neutral material, a single basic dipeptide which was Ala-Lys and a number of radioactive acidic peptides which were ninhydrin-positive. In addition there was a single acidic ninhydrin-positive peptide that was not radioactive that had a mobility relative to aspartic acid of 0.55. This peptide was eluted and 5% of it was hydrolyzed with 6 M HCl at 110\(^{\circ}\) for 24 hours in an evacuated sealed tube. Amino acid analysis of the dried hydrolysate revealed that it contained 3.9 \( \mu \)moles of cysteic acid and 4.6 \( \mu \)moles of threonine and traces of serine, glutamic acid, and glycine. The remainder of the peptide was separated into three aliquots which were freeze-dried. One of the aliquots was hydrolyzed in an evacuated sealed tube for 24 hours at 110\(^{\circ}\) with 300 \( \mu \)l of 6 M HCl. A second aliquot was hydrolyzed with 300 \( \mu \)l of 6 M HCl at 37\(^{\circ}\) for 30 hours in an evacuated sealed tube. The third aliquot was untreated. After freeze-drying, the three aliquots treated as described above were then applied, along a common origin, on Whatman No. 3MM and subjected to electrophoresis at pH 6.5 for 45 min at 3 kv. The ionogram was stained with the ninhydrin-cadmium reagent (17). A drawing which represents the relative intensity of ninhydrin-positive material observed on this ionogram is shown in Fig. 11. The aliquot of the dipeptide which was hydrolyzed at 110\(^{\circ}\) with 6 M HCl revealed cysteic acid and a neutral amino acid which is threonine. The partial acid hydrolysate revealed some neutral ninhydrin-positive material, and a new ninhydrin-positive spot with a mobility slightly greater than that of glutamic acid and a very faint ninhydrin-positive spot which migrated with the same mobility as the untreated peptide.

Based on Offord plots (22) in which the log of the electrophoretic mobility relative to aspartic acid is plotted against the log of the molecular weight of the peptide, we propose that the untreated pronase peptide has the composition shown in Structure II. Also, on the basis of an Offord plot, the ninhydrin-positive material in the partial acid hydrolysate which migrated slightly ahead of glutamic acid is the derivative shown by Structure I. The untreated peptide which is converted to cysteic acid and threonine by acid hydrolysis at 110\(^{\circ}\) for 24 hours, falls on an Offord plot with a net negative charge of \(-1\) if the molecular weight of the compound is based on Structure II. Similarly the ninhydrin-positive material with a mobility slightly greater than that of glutamic acid falls on an Offord plot with a net negative charge of \(-2\) if the molecular weight of the compound is based on Structure I.

These results suggest that tetrahydrophthalimide forms an adduct with Cys-149 when it inactivates the acyl phosphatase activity catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase and that the adduct is converted to cysteic acid on acid hydrolysis with 6 M HCl at 110\(^{\circ}\).
that the radioactive tryptic peptide isolated from Peak I of the DEAE-Sephadex column has the sequence and structure shown below.

\[
\text{Ile-Val-Ser-Asn-Ala-Ser-Cys-Thr-Thr-}
\]

where \( R \) is Structure III below and \( R' \) is \(-\text{CH}_2\text{COO}^-\).

The monoamide shown in Structure III accounts for the acidity of this peptide when it was subjected to electrophoresis at pH 6.5 and probably arose by hydroxysis of the \( \beta \)-hydroxythioether adduct of tetrahydrophthalimide with Cys-149 during the Sephadex purification steps which employed 0.05 M \( \text{NH}_4\text{OH} \) as solvent. This solvent would be sufficiently basic to hydrolyze the imide to the monoamide.

\[\text{DISCUSSION}\]

The experimental results presented clearly indicate that \[^{14}C\]-dimedone reacts covalently with sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase to form a covalent derivative of Cys-149. This inactivates the acyl phosphatase reaction catalyzed by the oxidized form of the enzyme. Since the covalent label is not removed by dithiothreitol, we suggest that the C-2 carbanion tautomer of dimedone reacts with the sulfenic acid at the active site of the enzyme as shown in Equation 3. If an enolate tautomer of dimedone had reacted with the sulfenic acid in a nucleophilic displacement reaction to form a sulfenyl ester, the label would have been removed by dithiothreitol and the dehydrogenase activity catalyzed by reduced glyceraldehyde 3-phosphate dehydrogenase would have been reactivated.

The results presented also show that the olefins 3-cyclohexene-1-carboxylate, dihydropyran, and tetrahydrophthalimide inactivate the acyl phosphatase reaction catalyzed by the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase. Since the addition of dithiothreitol to the acyl phosphatase inactivated with the olefins does not reactivate the dehydrogenase activity catalyzed by reduced glyceraldehyde 3-phosphate dehydrogenase, and since sulfenyl compounds in general (7, 8) form adducts with olefins, we suggest that the sulfenic acid at the active site of the acyl phosphatase forms addition products with the olefins during the activations described. Sequence analysis of the tryptic peptide which contains Cys-149 after the acyl phosphatase was inactivated with tetrahydrophthalimide strongly supports this hypothesis.

There are three other enzyme-catalyzed reactions that have been reported to be inactivated irreversibly by dimedone. Under certain conditions dimedone inactivates papain as reported by Morihara (23). Morihara showed that papain prepared by the method of Kimmel and Smith (24) which was inactive in the presence of reducing agents was inactivated when it was incubated with dimedone before the addition of activating thiols. Since the sulfhydryl group at the active site of papain (Cys-25) can be oxidized to a sulfenic acid by \( \text{H}_2\text{O}_2 \) (25) and that evidence has been presented that native, inactive papain is in part in the sulfenic acid form (26), it is possible that dimedone prevents the activation of Morihara's preparation of papain by reacting covalently with the sulfenic acid at the active site to form a covalent derivative of Cys-25.

Zeller (27) has shown that dimedone inactivates the non-flavin
amine oxidase isolated from beef plasma, an inactivation which we have recently repeated in our laboratory with the highly purified (specific activity >500) beef plasma non-flavin amine oxidase prepared by the method of Yasunobu and Smith (28). It has been postulated for years that the non-flavin amine oxidases contain pyridoxal phosphate or a related prosthetic group. This is based primarily on the fact that these amine oxidases are inactivated by carbonyl reagents. However, conclusive evidence that such a prosthetic group which contains an aldehyde moiety has never been presented (29). We have recently presented evidence that the sulfenic acid form of glyceraldehyde 3-phosphate dehydrogenase possesses a limited amine oxidase activity (30). Gorkin and his associates have shown that the oxidation of a sulfhydryl group in the mitochondrial amine oxidase converts the enzyme to an amine oxidase that is sensitive to carbonyl reagents and is specific for primary amines (31). These are the properties of the non-flavin amine oxidases. It is therefore tempting to suggest the following reaction mechanism for the non-flavin amine oxidases which hypothesizes a sulfenic acid and a sulfonamide intermediate on the reaction pathway as described by Equations 5 to 8.

\[
\begin{align*}
E SH + O_2 &\rightarrow E SOH + H_2O_2 \\
E SOH + H_2N-C-\beta &\rightarrow E S-C-\beta + H_2O \\
E ^{-} C-\beta &\rightarrow E^{+} + HN=C-\beta \\
\beta-C-NH + H_2O &\rightarrow \beta-C-O + NH_3
\end{align*}
\]

This reaction scheme is consistent with the ping-pong steady state kinetics exhibited by the non-flavin amine oxidase from pig plasma which suggests that two covalently modified forms of the enzyme are produced in the reaction sequence (32). The reaction scheme is also consistent with the products of the non-flavin amine oxidases which are H2O2, NH3, and the aldehyde (27). The non-flavin amine oxidases contain CuII which is necessary for activity (33). It is interesting that CuII in the presence of O2 oxidizes Cys-149 in glyceraldehyde 3-phosphate dehydrogenase to a sulfenic acid and that CuII in the presence of O2 oxidizes Cys-149 in glyceraldehyde 3-phosphate dehydrogenase to a sulfenic acid and converts the amine oxidase to one that is specific for primary amine and is no longer inactivated by paraglyine (31), a flavin-specific reagent.

Dimedone has also been reported to inhibit the oxidation of NADH by the mitochondrial electron transport chain by Weiss (34). Skidmore and Whitehouse have reported that dimedone and dimedone derivatives decrease the F:O ratio when rat liver mitochondria are treated with the compounds (35). Recent experiments performed in this laboratory have shown that the incubation of submitochondrial particles from beef heart with high phosphorylating activity prepared by the method of Hansen and Smith (30) with 7.5 mM dimedone for 2 min at 38°C inhibits ATP-driven reversed electron flow at coupling site I by 50%. Reversed electron flow was determined by the reduction of NAD4 by succinate which requires ATP as an energy source as described by Sanadi and his colleagues (37). Since we have postulated a scheme for chemical coupling in oxidative phosphorylation which postulates a sulfenyl carboxylate as a nonphosphorylated high energy intermediate (3), we are pursuing the possibility that these fairly high concentrations of dimedone are reacting covalently with a protein sulfenic acid or sulfenic acid derivative in the mitochondria and are thus inhibiting oxidative phosphorylation.

REFERENCES

1. ERICKING, R., and COLDWICK, S. P. (1969) J. Biol. Chem. 244, 4589-4599
2. ALLISON, W. S., and CONNORS, M. J. (1970) Arch. Biochem. Biophys. 136, 383-391
3. ALLISON, W. S., and BENITEZ, L. V. (1972) Proc. Natl. Acad. Sci. U. S. A. 69, 3004-3008
4. ALLISON, W. S., SWAIN, L. C., TRACY, S. M., and BENITEZ, L. V. (1973) Arch. Biochem. Biophys. 155, 400-404
5. KRAMER, I., and RACKER, E. (1955) Science 121, 319-321
6. KUHE, E. (1971) Synthesis 617-638
7. KUHE, E. (1971) Synthesis 505-506
8. KHALAQ, N. (1961) in Organic Sulfur Compounds (KHALAQ, N., ed) p. 375, Pergamon Press, New York
9. ELSON, P., and SEIDENSTICKER, Z. (1966) Acta Physiol. Acad. Sci. Hung. 9, 339-350
10. PARKER, J. D., and ALLISON, W. S. (1969) J. Biol. Chem. 244, 180-189
11. CARPENTER, F. H. (1967) Methods Enzymol. 11, 233
12. ALLISON, W. S., and KAPLAN, N. O. (1964) J. Biol. Chem. 239, 2140-2153
13. BAY, G. A. (1960) Anal. Biochem. 1, 279-285
14. LOWEY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J. (1951) J. Biol. Chem. 193, 265-275
15. MOORE, S., and STEIN, W. H. (1963) Methods Enzymol. 6, 819-831
16. LEE, T.-Y., STEIN, W. H., MOORE, S., and ELLIOTT, S. D. (1965) J. Biol. Chem. 240, 1143-1149
17. CAMPFIELD, L. R., and ANTEIS, C. B. (1963) Proteins 1, 311-375
18. HARRIS, J. I., and PERHAM, R. N. (1965) J. Mol. Biol. 13, 376-824
19. GRAY, R. W. (1967) Methods Enzymol. 11, 469-414
20. WOODS, K. R., and WANG, K.-T. (1967) Biochim. Biophys. Acta 133, 369-370
21. HARTLEY, B. S. (1970) Biochem. J. 119, 805-822
22. OFFORD, R. E. (1966) Nature 211, 872-873
23. MAAH, H. K. (1967) J. Biochem. (Tokyo) 62, 250-262
24. KIMMEL, J. R., and SMITH, E. L. (1957) Biochim. Biophys. Prep. 6, 61-67
25. ALLISON, W. S., and SWAIN, L. C. (1973) Arch. Biochem. Biophys. 155, 410-419
26. GLAZER, A. N., and SMITH, E. L. (1965) J. Biol. Chem. 240, 201-208
27. ZELLER, E. A. (1972) Advan. Biochem. Psychopharmacol. 5, 172-180
28. YASUNOBU, K. T., and SMITH, R. A. (1974) Methods Enzymol. 178, 988-704
29. KIMMEL, J. R., and SMITH, E. L. (1957) Biochim. Biophys. Acta 133, 369-370
30. ALLISON, W. S., BENITEZ, L. V., and JOHNSON, C. L. (1972) Advan. Biochem. Psychopharmacol. 166, 400-404
31. WATANABE, K., SMITH, R. A., INAMUSU, M., and YASUNOBU, K. T. (1972) Advan. Biochem. Psychopharmacol. 62, 107-117
32. ALLISON, W. S., BENITEZ, L. V., and JOHNSON, C. L. (1973) Biochim. Biophys. Acta 136, 2140-2153
33. SKIDMORE, I. F., and WHITEHOUSE, M. W. (1965) Biochem. J. 91, 547-550
34. HANSEN, M., and SMITH, A. L. (1964) Biochim. Biophys. Acta 166, 400-404
35. MATHUR, P., and WARSHAW, J. B., and SANADI, D. R. (1966) Arch. Biochem. Biophys. 117, 594-598
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