Mechanism of Action of Pyruvate Kinase

ROLE OF SULFHYDRYL GROUPS IN CATALYTIC ACTIVITY AS DETERMINED BY DISULFIDE INTERCHANGE

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SUMMARY

Evidence is presented that four sulfhydryl residues play a critical role in the activity of pyruvate kinase. Modification of the four most reactive sulfhydryl groups by 5,5'-dithiobis-(2-nitrobenzoate) leads to a disulfide interchange reaction in which a chemically modified but catalytically active enzyme is produced. This form, which contains 5-thio-2-nitrobenzoate in disulfide linkage to cysteine residues, is stable at low pH or in the presence of phosphoenolpyruvate and metal ions. At neutral pH and in the absence of these compounds, the thionitrobenzoate-enzyme undergoes an intramolecular disulfide interchange involving four additional but essential sulfhydryl residues to liberate thionitrobenzoate with the formation of cystine residues. The resulting form of pyruvate kinase is catalytically inactive.

Evidence was recently presented from studies on the inactivation of rabbit muscle pyruvate kinase by 2,4,6-trinitrobenzenesulfonate that four lysyl ε-amino groups are essential for catalytic activity and that these groups are involved in the binding of ADP by the enzyme (2). The involvement of amino groups is also supported by the finding of Johnson and Deal (3) that the enzyme is inactivated by pyridoxal 5'-phosphate, due to Schiff base formation. Evidence exists suggesting that sulfhydryl groups may also be required for activity, since the enzyme is known to be inhibited by p-mercuribenzoate and certain other sulfhydryl-binding agents (4-8).

In the present paper evidence is given for the occurrence of the following reactions when pyruvate kinase is treated with DTNB:

In these equations $E$ represents a subunit of pyruvate kinase. In Reaction $a$ the native enzyme undergoes a primary disulfide interchange with DTNB to yield a catalytically active TNB-enzyme with the liberation of free TNB. In Reaction $b$ the modified enzyme undergoes a secondary intramolecular disulfide interchange to give a catalytically inactive disulfide-enzyme with the formation of additional free TNB. It should be noted that rabbit muscle pyruvate kinase contains four $N$-acetyl groups (9) and is composed of four apparently identical polypeptide chains or subunits, as indicated by sedimentation studies in the presence of dissociating agents (9, 10) and by disc gel electrophoresis and peptide mapping (9). The present work, which shows that each of the four enzyme subunits contains a sulfhydryl group which is essential for catalytic activity, thereby indicates that the subunits may be identical in function as well as in structure.

EXPERIMENTAL PROCEDURE

Materials

Pyruvate kinase was prepared from frozen rabbit skeletal muscle by the method of Tietz and Ochoa (11). After two or three recrystallizations from 0.02 M imidazole buffer, pH 7.0, containing $1.0 \times 10^{-4} M$ EDTA, the enzyme had a specific activity of

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about 330 units per mg of protein and was homogeneous as judged by sedimentation and electrophoretic measurements (9). The enzyme was stored at 4° as an ammonium sulfate suspension at a concentration of about 20 mg of protein per ml and, before use in the experiments to be described, was dialyzed for 4 hours at 4° against 500 volumes of 0.1 M Tris-chloride buffer, pH 7.4, with a change of buffer at 2 hours. Protein concentrations were determined spectrophotometrically with an extinction coefficient of 0.64 mg⁻¹ cm⁻¹ (12).

DTNB, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide, and disodium EDTA were obtained from Sigma, Sephadex G-25 from Pharmacia, bromopyruvate from Aldrich, and other materials as described previously (2). All solutions were prepared with glass-distilled water.

Methods

Assay of Pyruvate Kinase—The catalytic activity of pyruvate kinase was determined at 30° in 0.1 M Tris-chloride buffer, pH 7.4, as described previously (13), by the spectrophotometric method of Biehl and Pfeiderer (12) as modified by Tietz and Ochoa (11). The loss in absorbance due to the oxidation of DPNH was measured at 340 nm.

Determination of Enzyme Inactivation by Sulphydryl-binding Agents—Pyruvate kinase in 0.1 M Tris-chloride buffer was incubated with DTNB or other inhibitors at 30°, and small aliquots of the reaction mixture were removed at intervals, immediately diluted with 0.05 M Tris buffer, pH 7.4, and assayed for residual catalytic activity as described. In such experiments the dilution was sufficiently great to stop the action of the sulphydryl-binding agents on the enzyme.

Determination of Reaction of Enzyme Sulphydryl Groups with 5,5′-Dithiobis(2-nitrobenzoate)—Solutions of pyruvate kinase in 0.1 M Tris buffer, pH 7.4, were incubated at 30° for 5 min prior to the addition of DTNB. The release of TNB was determined at the same temperature in a spectrophotometer equipped with a Gilford multiple sample absorbance recorder. The molar extinction coefficient of TNB at 412 nm is 13,600 (14). The enzyme was omitted in control experiments.

Amino Acid Analysis—The enzyme preparations to be analyzed were thoroughly dialyzed against glass-distilled water and then taken to dryness by lyophilization in test tubes which had previously been washed with nitric acid to remove metal ions. About 2 ml of 6 N HCl were added per mg of protein, and the sample was frozen in a Dry Ice-acetone bath, flushed with nitrogen, and degassed with a vacuum pump. This procedure was repeated at least twice, and the tubes were then sealed under a vacuum and kept at 110° for 24 hours. The resulting solutions were concentrated in a rotary evaporator at 37°, and the dry residues were dissolved in 0.2 M sodium citrate buffer, pH 2.2. Amino acid analyses were carried out by the method of Crestfield, Moore, and Stein (15) with a Beckman 120C amino acid analyzer equipped with an Infotronics automatic digital integrator. The number of residues of a particular amino acid was determined relative to the aspartate or alanine content of the protein as described previously (9).

RESULTS

Cysteine and Cystine Content of Pyruvate Kinase—The total number of cysteine residues in pyruvate kinase was previously estimated to be 36 by treatment of the protein with DTNB in the presence of guanidine or sodium dodecyl sulfate (9). Since the enzyme contains 4 apparently identical subunits (9, 10), it is concluded that 9 cysteine residues are present in each. Cysteic acid was obtained in the amount of 10.6 moles per mole of subunit when the enzyme was acid-hydrolyzed in the presence of dimethyl sulfoxide by the method of Spencer and Wold (16) and in the amount of 11.4 moles per mole of subunit when the enzyme was submitted to performic acid oxidation by the method of Moore (17), giving an average value of 11.0. Since in these methods the cysteic acid is derived from both cysteine and cystine, it is apparent that in the native enzyme 1 cystine residue may be present per subunit.

Inactivation by Sulphydryl-binding Agents—In preliminary experiments iodoacetamide, bromopyruvate, N-ethylmaleimide, and N-dimethylaminonitrophenylmaleimide were all found to inactivate pyruvate kinase; the pseudo-first order rate constants for enzyme inactivation at 30° in the presence of these reagents were 0.006, 0.02, 0.14, and 1.05 min⁻¹, respectively. These values were determined with 2.0 × 10⁻⁷ M enzyme in 0.1 M Tris buffer, pH 8.2, containing 0.01 M sodium acetate, and with a molar ratio of inhibitor to enzyme of 250, except with N-dimethylaminonitrophenylmaleimide, where the ratio was 125.

That extensive loss of sulphydryl groups had occurred was shown for two of the modified proteins. After acid hydrolysis of the N-dimethylaminonitrophenylmaleimide-treated protein, 12 to 20 residues of S-(1,2-dicarboxyethyl)-L-cysteine were found, and after acid hydrolysis of the bromopyruvate-treated enzyme following dialysis against dilute hydrogen peroxide (18), a similar amount of carbamethylycysteine was identified.

Because of the specificity with which DTNB modifies the sulphydryl groups of pyruvate kinase, the nature of the reaction was studied in detail. The kinetics of enzyme inactivation by this inhibitor at pH 7.4 is shown in Fig. 1. In these experiments the enzyme was incubated with various concentrations of DTNB, and aliquots were removed at intervals and diluted sufficiently to halt the inactivation reaction. The catalytic rates determined with the DTNB-treated enzyme, like those obtained with the native enzyme, were found to be constant for at least 5 min and to be directly proportional to the protein concentration. In all cases the inactivation reaction was characterized by an initial lag phase, the cause of which will be indicated below. The subsequent more rapid reaction was pseudo-first order with respect to enzyme concentration at all concentrations of DTNB tested. The apparent first order rate constant was 0.24 min⁻¹ at the highest level of the inhibitor, which was present in 300-fold molar excess. In other similar experiments it was shown that the enzyme was completely inactivated by DTNB at longer incubation times and that the plots of log percentage of residual activity as a function of time were linear (beyond the initial lag phase) to the point where all activity was lost.

The apparent order of the reaction with respect to the inhibitor was determined from the data in Fig. 1 by a graphic procedure used previously for studying the reaction of trinitrobenzene-1-sulfonate with pyruvate kinase (2). As shown in Fig. 2, a plot of the log of the reciprocal of the half-time of the inactivation reaction as a function of the log of the inhibitor concentration gave a straight line. The slope, which corresponds to the order of the reaction, i.e. the number of molecules of inhibitor reacting with each active unit of the enzyme to produce an inactive enzyme-inhibitor complex, was found to be 0.43. This fractional value suggests that the inactivation reaction may be a separate event from the initial reaction of the inhibitor with the enzyme. Thus, the formation of a catalytically active enzyme-inhibitor complex (Reaction a), as will be documented below, may be followed either by a step in which the inhibitor is removed to give an inactive form of the enzyme (Reaction b), or by a step in...
a modified but active form of the enzyme in Reaction a, followed bound to the enzyme, with a statistical distribution accounting Occurrence of both pathways (Reactions b and c) might give the which combination with one or more additional molecules of occurrence fractional value for the order of the reaction. This method was used by Levy et al. (19), who showed that at least 3 molecules of dinitrophenol are bound to an active unit of myosin during inactivation, and by Scrutton and Utter (20), who obtained a value of 1.4 for the inactivation of pyruvate carboxylase by avidin and proposed that 1 or more molecules of avidin are bound to the enzyme, with a statistical distribution accounting for the observed order. It may be noted that the formation of a modified but active form of the enzyme in Reaction c, followed by the inactivation step in Reaction b, could account for the biphasic nature of the reaction shown in Fig. 1.

Protection by Substrates and Metals against Enzyme Inactivation—The effect of substrates and metal activators on enzyme inactivation by DTNB is shown in Table I. The various compounds, with the exception of NH4Cl, were added at the concentrations employed in the usual pyruvate kinase assay system. When tested individually, potassium ions and ADP were ineffective, whereas magnesium ions and P-enolpyruvate each provided significant protection. P-enolpyruvate was more effective in the presence of K+ or Mg2+ ions and in the presence of both metal activators it provided complete protection against inactivation by DTNB. In contrast, the combination of ADP, K+ ions, and Mg2+ ions was no more effective than Mg2+ ions alone. The ability of some of the substrates and metals to protect against inactivation suggests that DTNB inhibits by reacting with a sulfhydryl residue located in the active center of the enzyme.

The protective effect of ammonium chloride is not a function of ionic strength alone and is apparently not due to the chloride ion, for at similar concentrations tetramethylammonium chloride had no significant effect on the rate of inactivation of the enzyme by DTNB. In other experiments potassium and ammonium ions at concentrations greater than 0.3 M were shown to have a similar protective effect, whereas at lower concentrations ammonium ions gave protection and potassium ions increased the rate of inactivation by DTNB. Since the catalytic activity of pyruvate kinase is known to be supported by either K+ or NH4+ ions, but not by tetramethylammonium ions (21), it appears that the ability of a monovalent metal cation to function in the catalytic activity and to protect against DTNB are closely related.

Kinetics of Enzyme Reaction with 5,5'-Dithiobis(2-nitrobenzoate) Measured by Liberation of TNB—The rate of reaction of DTNB with enzyme sulfhydryl groups was determined by the release of TNB, as shown in Fig. 3. The reaction was significantly slower when ammonium ions were included in the reaction mixture (Curve B), in accord with the effect of NH4+ ions in decreasing the rate of enzyme inactivation, as mentioned above. It is evident that no lag occurred in the early phase of TNB libera-
tion, in contrast to the results obtained when the early phase of enzyme inactivation was examined. The results obtained show that at least 28 to 30 sulfhydryl residues were modified, either in the presence or absence of ammonium ions. Since beyond this point the solutions became turbid due to protein denaturation, the end point of the reaction could not be determined by this method.

The kinetics of sulfhydryl modification in the presence of substrates and metal activators is shown in Fig. 4. In all cases, TNB liberation was characterized by a rapid initial phase which was not appreciably affected by the presence of the added components. Pronounced differences were noted, however, beyond about 10 min. Whereas ADP and K⁺ ions either alone or together gave no significant protection of sulfhydryl groups, as judged by TNB liberation, P-enolpyruvate either alone or in the presence of Mg⁺⁺ ions prevented the loss of all but about 4 sulfhydryl residues, even at 60 min. Intermediate effects were noted with Mg⁺⁺ ions, either alone or with K⁺ ions, or ADP, or both K⁺ ions and ADP also present. In general, those compounds which were most effective in protecting the enzyme against inactivation were most effective in retarding the reaction of DTNB with sulfhydryl residues.

Correlation of Loss of Activity with Extent of Sulfhydryl Group Modification—Studies were next undertaken to determine whether the inhibition by DTNB could be correlated more precisely with the loss of a specific number of sulfhydryl groups. The results are shown in Fig. 5. In such experiments the initial molar ratio of DTNB to enzyme was 4, so that DTNB and enzyme subunits were present in equal concentrations. Under these conditions the rate of the reaction became very slow as it neared completion, and it was necessary to extrapolate the data to show that complete inactivation of pyruvate kinase is accompanied by the release of 8 moles of TNB per mole of enzyme, or 2 moles per mole of enzyme subunit. Such results are entirely in accord with the over-all stoichiometry predicted from the sum of Equations a and b given above.

As would be expected from the evidence already presented, quite different results were obtained when a similar experiment was carried out with P-enolpyruvate, K⁺, and Mg⁺⁺ ions present in the reaction mixture. The effect of DTNB on catalytic activity and sulfhydryl group modification as a function of time under these conditions is given in Fig. 6. TNB release occurred relatively rapidly until about 4 moles had been liberated per mole of enzyme.

% INHIBITION

MOLES OF TNB RELEASED PER MOLE OF ENZYME

Fig. 5. Extent of inhibition of pyruvate kinase as a function of TNB liberation. The enzyme (2.0 X 10⁻⁶ M) in 0.1 M Tris buffer, pH 7.8, containing either 0.75 M (O) or 1.95 M NH₄Cl (△) was incubated at 30° with DTNB. The initial molar ratio of DTNB to enzyme was 4. At intervals the TNB concentration was determined from the absorbance at 412 nm, and aliquots of the reaction mixture were removed, diluted, and assayed for residual catalytic activity.

Fig. 6. Loss of activity and TNB release as a function of time upon treatment of pyruvate kinase with DTNB in the presence of protective agents. The enzyme (3.2 X 10⁻⁶ M) in 0.1 M Tris buffer, pH 7.4, containing 0.1 M KCl, 4.0 X 10⁻⁵ M MgCl₂, and 1.0 X 10⁻⁴ M P-enolpyruvate, was incubated briefly at 30° before the addition of DTNB. The initial molar ratio of DTNB to enzyme was 4. The activity of the modified enzyme is given relative to that of the untreated enzyme as 100%. 

![Graph](http://www.jbc.org/)
of enzyme and was then characterized by a very slow, linear phase. Extrapolation of the slow phase of the curve to the vertical axis indicated that the more rapid phase resulted in the modification of about 3.6 sulfhydryl residues. The catalytic activity of the enzyme was protected by the presence of P-enolpyruvate and metal ions, in accord with data already presented. Almost full activity remained at 120 min, when almost 4 moles of TNB had been released per mole of protein, and thereafter the activity was lost only slowly, remaining as high as 80% even at 8 hours. In addition, the unexpected observation was made that during the modification of up to two sulfhydryl residues the enzyme reached an activity about 40% greater than that of the untreated protein.

These findings (Figs. 5 and 6) clearly indicate that the modification of four sulfhydryl groups by DTNB in the presence of substrate and metal ions yields a protein which retains catalytic activity, but that the modification of four additional sulfhydryl groups gives a different species which is devoid of activity. Since only 4 moles of DTNB were added per mole of enzyme, the release of a total of 8 moles of TNB in the absence of protective agents (Fig. 5) must have involved disulfide interchange between DTNB and the enzyme, followed by a second disulfide interchange within the resulting TNB-enzyme to yield the additional amount of free TNB.

Properties of TNB-Enzyme—Initial attempts to separate the catalytically active TNB-enzyme from the protective agents (P-enolpyruvate and metal ions) by gel chromatography at pH 7.5 were unsuccessful because the protein underwent a rapid loss in activity, accompanied by the release of free TNB. Since disulfide interchange is generally less rapid at low pH (22), the experiment was repeated with a column equilibrated with acetate buffer, pH 4.9, with the results shown in Fig. 7. At this pH the TNB-enzyme, which appeared in the void volume and was well separated from free TNB, was found to be stable for at least 1 hour in the absence of substrate and metal ions. The TNB-enzyme concentration was determined by amino acid analysis, and the extinction coefficient at 280 nm was calculated to be 0.61 ml mg\(^{-1}\) cm\(^{-1}\). Clearly, therefore, the introduction of TNB groups alters the extinction coefficient, which is 0.54 ml mg\(^{-1}\) cm\(^{-1}\) for the normal enzyme. The enzyme in the peak tube, which had a specific activity 15% greater than the native enzyme, was used in the experiments to be described. The spectrum of the TNB-enzyme, which is given in Fig. 8, is characterized by absorption maxima at 324 and 280 nm. The lack of significant absorbance at 412 nm indicates the absence of free TNB. The peak at 324 nm is also characteristic of DTNB, evidence is presented below, however, for the absence of bound DTNB in this enzyme preparation.

Conversion of TNB-Enzyme to Disulfide-Enzyme—TNB-enzyme was prepared by gel chromatography in the manner described. The pH was then adjusted to 7.5, and at intervals the amount of TNB liberated was determined spectrophotometrically, and the loss in activity was determined by assay of aliquots of the solution. The data in Fig. 9 show that the inactivation of the enzyme is directly related to TNB release and that all activity is lost when about 4 moles of TNB have been released per mole of TNB-enzyme.

The effect of pH on the rate of inactivation of the enzyme during the conversion of TNB-enzyme to disulfide-enzyme is shown in Fig. 10. The rate increased with increasing pH over the range studied, and the first order rate constants varied from 0.05 min\(^{-1}\) at pH 6.7 to 0.86 min\(^{-1}\) at pH 8.5. These results are taken to mean that an essential sulfhydryl residue with a pK of about 7.5 undergoes the disulfide interchange with the TNB-enzyme disulfide group. A lag was not seen in the plots of log percentage of residual activity as a function of time in the conversion of TNB-enzyme to disulfide-enzyme, in sharp contrast to the results obtained earlier when DTNB was added to the native enzyme (cf. Fig. 1). In the earlier experiments, as is now clear, the results were a composite of the conversion of pyruvate kinase to the active TNB-enzyme and the further transformation of the latter to the disulfide-enzyme (Reactions a and b).

In order to obtain additional evidence that the inactivation of the TNB-enzyme is associated with the formation of a new cystine disulfide bond in the protein, the sulfhydryl content of the disulfide-enzyme was determined. In confirmation of earlier work (9), treatment of the unmodified pyruvate kinase by DTNB in the presence of 3% sodium dodecyl sulfate indicated the

![Fig. 7. Isolation of TNB-enzyme by column chromatography](http://www.jbc.org/)

A solution of 1.7 mg of pyruvate kinase in 0.98 ml of 0.08 M Tris buffer, pH 7.4, containing 0.1 M KCl, 4.0 \times 10^{-3} M MgCl\(_2\), and 1.0 \times 10^{-3} M P-enolpyruvate, was incubated at 22° for a few minutes. A concentrated solution of DTNB (0.02 ml) was then added so that the final molarity was 25 times that of the pyruvate kinase, and the release of TNB was followed with time. As soon as 4 moles of TNB had been liberated per mole of enzyme, the reaction mixture was applied at room temperature to a Sephadex G-25 column (0.9 \times 26 cm) which had been equilibrated with 0.05 M potassium acetate buffer, pH 4.9. The column was washed with the buffer, and the modified protein appeared in the void volume. The flow rate was 60 ml per hour, and the fraction volume was 1.8 ml.

![Fig. 8. Spectrum of TNB-enzyme](http://www.jbc.org/)

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presence of 35.8 moles of sulfhydryl groups per mole of protein.
A similar value, 35.6, was obtained with 4,4′-dithiopyridine (23)
in place of DTNB. In contrast, when the disulfide-enzyme was
exposed to DTNB in the presence of sodium dodecyl sulfate, only
27.5 moles of sulfhydryl groups were found to be present. These
results indicate that about eight sulfhydryl groups, or two per
subunit, are lost during the formation of the disulfide-enzyme.

Conversion of Disulfide-Enzyme to Catalytically Active Enzyme
by Treatment with Dithiothreitol—As further evidence that the
inactive enzyme contains a new disulfide residue, it was treated
with dithiothreitol as a reducing agent with the results shown in
Fig. 11. In this experiment the disulfide-enzyme was generated
on the TNB-enzyme with the anticipated release of free TNB
and loss of about 98% of the catalytic activity. Then, upon the
addition of 0.01 M dithiothreitol at 17 min, catalytic activity was
rapidly restored, reaching a maximum of about 90% of the origi-
nal activity. In other experiments, similar results were obtained
with 0.01 M mercaptoethanol. Since no further increase in the
level of free TNB occurred upon the addition of dithiothreitol,
it is evident that the disulfide-enzyme contains neither bound
DTNB nor covalently bound TNB.

Comparison of Sedimentation of Native Enzyme and Disulfide-
Enzyme—Sucrose density gradient sedimentation was undertaken
to determine whether the new disulfide bond in the inactivated
enzyme occurs within each subunit or between subunits, with
the results presented in Fig. 12. The disulfide-enzyme (pre-
pared from the isolated TNB-enzyme by adjusting the pH of the
solution to 7.8) and the native enzyme were each exposed to 4 M
urea. This treatment is known to dissociate the native protein
into the monomeric form, as shown previously (9, 10). Since
the urea treated disulfide enzyme has, within experimental error,
the same sedimentation properties as the similarly treated native
enzyme, as shown in the figure, it is apparent that the subunits in
the disulfide-enzyme are readily dissociated and are therefore
not linked covalently by disulfide bonds. Accordingly, it is
concluded that the inactivation process leads to the formation of
a disulfide bond within each polypeptide chain. In other experi-
ments for which the data are not given, the TNB-enzyme was
shown to have the same sedimentation properties in the absence
of urea as does the native enzyme. Presumably, therefore, the
TNB-enzyme also possesses a tetrameric structure.

Protection by Various Compounds against Inactivation of TNB-
Enzyme—The ability of various compounds to protect the TNB-
enzyme against inactivation due to formation of the disulfide-
enzyme at pH 7.8 is indicated in Table II. When tested
individually, ADP and K+ ions gave no significant protection,
whereas P-enolpyruvate and Mg++ ions were partially effective.
A combination of ADP and Mg++ ions strongly protected the
enzyme, and the further addition of K+ ions enhanced the effect.
Complete protection of catalytic activity was achieved only in
the presence of P-enolpyruvate, Mg+++, and K+ ions; in other

FIG. 10 (right). Effect of pH on the rate of conversion of TNB-
enzyme to disulfide-enzyme as judged by enzyme inactivation. The
TNB-enzyme in acetate buffer was mixed with various Tris
buffers to give the pH values indicated. The final concentrations
were: TNB-enzyme, 1.67 × 10⁻⁷ M; potassium acetate, 2.5 × 10⁻³ M;
and Tris buffer, 0.095 M. The pH values were determined in
the final complete reaction mixtures. Aliquots were taken at
intervals for the determination of catalytic activity, and the
resulting data were plotted as in Fig. 1 to determine the reciprocal
of the half-time of the reaction.

FIG. 11. Regeneration of active enzyme from disulfide-enzyme.
The TNB-enzyme in acetate buffer, pH 4.9, was adjusted to pH 8.1
by the addition of Tris base, and loss in activity and TNB release
due to formation of the disulfide-enzyme were followed with time
at 30°. The initial concentrations were: TNB-enzyme, 1.76 ×
10⁻⁷ M; potassium acetate, 2.5 × 10⁻³ M; and Tris buffer, 0.095 M.
The solution containing the disulfide-enzyme was made 0.01 M
with respect to dithiothreitol at 17 min, as indicated by the arrow.

FIG. 12. Sucrose density gradient sedimentation patterns.
The native enzyme in 0.1 M Tris buffer (C), pH 7.9, and the disul-
finite-enzyme in the same buffer containing 0.02 M potassium acetate
(A) were each incubated in 4.0 M urea at 0° for 1 hour, and a 0.2-
ml aliquot (containing 300 or 350 µg of protein, respectively),
was removed and layered on top of 5.0 ml of a 5 to 15% sucrose gradient
according to the method of Martin and Ames (24), except that 4.0
M urea was present. Centrifugation was carried out in a Spinco
model L ultracentrifuge with an SW 65 rotor at 65,000 rpm for 9
hours at 4°. The fractions (3 drops each) were diluted in water,
and the protein content was measured by the method of Lowry
et al. (25). The bottom of the gradient is on the left.
TABLE II
Protection by various compounds against inactivation of 5-thio-2-nitrobenzoate-enzyme by disulfide interchange

To a solution of the various compounds in 0.1 M Tris buffer, pH 7.8, at 30°C, the TNB-enzyme in acetate buffer, pH 4.9, was added, and after incubation of the mixture for 10 min at the same temperature aliquots were removed and assayed for residual activity. The final pH was 7.5 and the concentrations were as follows: TNB-enzyme, 3.3 X 10^-4 M; potassium acetate, 0.01 M; P-enolpyruvate, 1.0 X 10^-3 M; ADP or ATP, 5.0 X 10^-3 M; KCl, 0.1 M; MgCl₂, 4.0 X 10^-3 M; potassium lactate or pyruvate, 0.01 M; and KHCO₃, 0.17 M.

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</tr>
<tr>
<td>KCl</td>
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</tr>
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The data presented are in accord with Reactions a and b as given above. All forms of the enzyme are shown as tetramers in these reactions, but this is known with certainty only for the native enzyme; the TNB-enzyme has similar sedimentation properties, but the state of aggregation of the disulfide-enzyme has not been studied. Evidence was obtained showing that 8 molecules of TNB are liberated and 8 cysteine residues are lost when the native enzyme is converted to the TNB-enzyme, however, remains to be established.

DISCUSSION

The data presented are in accord with Reactions a and b as given above. All forms of the enzyme are shown as tetramers in these reactions, but this is known with certainty only for the native enzyme; the TNB-enzyme has similar sedimentation properties, but the state of aggregation of the disulfide-enzyme has not been studied. Evidence was obtained showing that 8 molecules of TNB are liberated and 8 cysteine residues are lost in the over-all inactivation reaction, that 4 molecules of TNB are liberated when the native enzyme is converted to the TNB-enzyme in the presence of agents which stabilize the latter protein, that 4 molecules of TNB are released when the isolated TNB-enzyme is converted to the disulfide-enzyme, and that the original activity is largely restored when the inactivated enzyme
and P-enolpyruvate. Furthermore, the disulfide-enzyme catalytically altered during the formation of the catalytically inactive disulfide-enzyme. The conclusion that four active centers to occur with the native enzyme (34). These more recent findings are present in the enzyme is supported by our previous studies on the role of four lysyl-e-amino groups in the binding of ADP to the enzyme (2) and is also supported by the work of others who have shown that the enzyme contains four binding sites for monovalent metal cations (35), divalent metal cations (36, 37), and P-enolpyruvate (34).

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