Chemical Modification of the Active Site Sulphhydril Group of Saccharopine Dehydrogenase (L-Lysine-forming)*

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Saccharopine dehydrogenase (ε-N-(L-glutaryl-2)-L-lysine:NAD oxidoreductase (L-lysine-forming) EC 1.5.1.7) is inhibited by a variety of sulphhydril reagents. Although p-chloromercuribenzoate and 5,5’-dithiobis(2-nitrobenzoate) react with three sulphhydril groups present in the enzyme, reaction with one reactive sulphhydril group leads to complete loss of catalytic activity. Amino acid analysis of the enzyme carboxymethylated by iodoacetate shows the formation of 1 eq of S-carboxymethylcysteine. The loss of one sulphhydril group titratable with 5,5’-dithiobis(2-nitrobenzoate) after carboxymethylation confirms the site of modification as a cysteine residue. The inhibition by iodoacetate follows second order kinetics, with a rate constant of 0.2 mm-1 min-1 at pH 8.5 and 24°C. Modification of 1 cysteine residue results in total inactivation. The inhibition by iodoacetate is protected by the coenzyme (or analogs) and the coenzyme plus substrate (or analog). The effectiveness of protection by these compounds closely parallels their affinities for the enzyme, indicating that the modification occurs at the active site residue. The modification of the cysteine residue appears to result in a loss of ability to bind the coenzyme. Among alkylating agents tested, N-ethylmaleimide and iodoacetamide are stronger inhibitors than iodoacetate, as evidenced by their second order rate constants for inhibition.

Saccharopine dehydrogenase (ε-N-(L-glutaryl-2)-L-lysine:NAD oxidoreductase (L-lysine-forming) EC 1.5.1.7), which catalyzes the reversible cleavage of saccharopine to lysine and α-ketoglutarate in the presence of a pyridine nucleotide coenzyme, has recently been purified to homogeneity from baker’s yeast (1). The yeast enzyme is a monomeric protein with a molecular weight of 39,000 and contains 3 cysteine residues/mol. No disulfide bond is present (1).

A preliminary study in this laboratory has shown that the enzyme is inhibited by p-chloromercuribenzoate and iodoacetate. The irreversible inhibition by iodoacetate was protected by an excess amount of the coenzyme, suggesting that the modified residue occurs at or near the active site (1). The present investigation was undertaken to identify the residue involved in the inactivation and to learn about the functional role played by this residue in the enzymic reaction.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—NAD*, NADH (Oriental Yeast Co., Osaka); NADPH, mersalyl acid, o-iodosobenzoic acid, ADP, ATP (Sigma); AMP (Kyowa Hakko Co., Tokyo); 5,5’-dithiobis(2-nitrobenzoic acid), p-chloromercuribenzoic acid, N-ethylmaleimide (Nakarai Chemicals, Kyoto); N-butylmaleimide (Nutritional Biochemical Corp.) were purchased from sources indicated. Iodoacetate acid (Merck) was recrystallized from chloroform. Saccharopine was prepared enzymatically as described previously (2). [1H]NAD+ (5 Ci/mmol), [U-14C]α-ketoglutaric acid (200 mCi/mmol), L-[[3H]cysteine (60 Ci/mmol) were obtained from New England Nuclear, and L-[14C]iodoacetic acid (48 mCi/mmol) from Amersham/Searle. [3H]NADP was prepared by the method of [H]NADP* with ethanol and alcohol dehydrogenase essentially as described by Dalziel (3). A freshly prepared [1H]NADP had a specific radioactivity of 1 to 2 × 10^6 cpm/μmol, and the A_260/A_250 ratio of 2.4 to 2.5. Preparations of NADH exceeding this ratio were not used in the experiments.

**Enzyme Preparation and Assay**—Saccharopine dehydrogenase was purified from baker’s yeast (Oriental Yeast Co., Osaka) as previously described (1). The saccharopine dehydrogenase activity was determined by the rate of decrease of NADH at 340 nm in the direction of reductive condensation of lysine and α-ketoglutarate (1).

**Equilibrium Dialysis**—The binding capacities of the enzyme for various ligands were analyzed by the equilibrium dialysis method. The equilibrium dialysis was performed in an apparatus containing eight sets of dialysis cells. Each cell was separated into three chambers (10 mm deep and 5 mm wide) by Visking 36/32 dialysis tubing pretreated with 5% Na_2CO_3, 10 mM EDTA at 100°C for 5 min (4). For NADH-binding experiments, 0.1 ml of 12.8 μM or 10.2 μM enzyme solutions in 0.1 mM Tris/HCl buffer, pH 8.5, were placed in the center chambers, and 0.1 ml of [3H]NADH solutions in the same buffer were added to the outer chambers. The whole apparatus was agitated gently for about 16 h at 24°C. During this period, essentially no loss in enzymic activity was observed. Samples (50 μl) from each chamber were transferred to vials containing 9 ml of a scintillation fluid (toluene, 607 ml, Triton X-100, 333 ml, 2,5-diphenyloxazole, 5 g, 1,4-bis(6-phenylxazolyl)benzene, 0.4 g) and 0.95 ml of water. The composition was determined according to the method of Lowry et al. (6) with a Beckman model 120 amino acid analyzer.

**Amino Acid Analysis**—The dehydrogenase (0.3 nmol) in 0.5 ml of 0.5 M Tris/HCl buffer, pH 9.3, was incubated with 10 μl of 10 mM [2-14C]iodoacetate (8.5 × 10^6 cpm/μmol). At appropriate times, aliquots were removed for measurements of the saccharopine dehydrogenase activity and radioactivity. The radioactivity incorporated was determined by the filter paper disc method as described by Bollum (5). Aliquots (50 μl) of the reaction mixture were plated on Toyo Roshi No. 51 filter paper discs, and the discs were immediately immersed in 10% trichloroacetic acid to stop the reaction. The filter paper discs were then washed 5 times with 100-ml portions of 10% trichloroacetic acid, twice with 50-ml portions of ethanol, and finally with ethyl ether. They were counted in a scintillation spectrometer using a toluene scintillation mixture.

**Amino Acid Analysis**—The dehydrogenase (0.3 mg) which had been inactivated more than 95% by the reaction with 20-fold molar excess of iodoacetate at pH 9.3, was dialyzed against 2 l of water in the dark at 4°C. After lyophilization and hydrolysis, its amino acid composition was determined according to the method of Crestfield et al. (6) with a Beckman model 120 amino acid analyzer.

**Other Determinations**—Protein concentration was determined by the method of Lowry et al. (7). A molecular weight of 39,000 for...
saccharopine dehydrogenase (1) was used in all calculations. pH determinations were made with 0.5 M buffer solutions at 24°C.

RESULTS

Effect of Sulfhydryl-directed Reagents on Catalytic Activity of Saccharopine Dehydrogenase

The saccharopine dehydrogenase activity was inhibited by a variety of sulfhydryl-directed reagents (Table I). As the table shows, the mercury compounds were the most potent inhibitors, and complete loss in activity occurred instantaneously on addition of the reagents. Among alkylating agents, substituted maleimides were more active than iodoacetamide or iodoacetate.

Reaction with p-Chloromercuribenzoate

When saccharopine dehydrogenase was titrated with p-chloromercuribenzoate in 0.1 M potassium phosphate buffer, pH 6.8, containing 0.45 M (NH₄)₂SO₄, an increase in absorbance at 250 nm due to mercaptide formation occurred almost instantaneously. As depicted in Fig. 1, the maximal increase in absorbance was found to be equivalent to three sulfhydryl groups reacted/mol of enzyme, as calculated from the molar extinction coefficient of 7.8 × 10⁵ M⁻¹ cm⁻¹ (8). The enzymic activity was completely lost before the reaction of three sulfhydryl groups. Extrapolation of the linear portion of the plot to zero activity showed that the reaction with one thiol group caused a complete loss in activity. Thus, although three sulfhydryl groups present in the enzyme react very rapidly with p-chloromercuribenzoate, the one essential for catalytic activity is more susceptible to the mercurial than the other two. The inhibition by p-chloromercuribenzoate was reversible: when the p-chloromercuribenzoate-treated enzyme was dialyzed overnight against 0.1 M potassium phosphate buffer, pH 6.8, containing 1 mM dithiothreitol, 83.2% of original enzyme activity was recovered. An excess of the coenzyme and substrates afforded no protection against inhibition by p-chloromercuribenzoate.

Reaction with 5,5′-Dithiobis(2-nitrobenzoate)

Unlike p-chloromercuribenzoate, the reaction of 5,5′-dithiobis(2-nitrobenzoate) was rather slow even in the presence of (NH₄)₂SO₄ which accelerated the reaction. Fig. 2 shows that the enzymic activity is completely lost on modification of 1 sulfhydryl residue. The inhibition by 5,5′-dithiobis(2-nitrobenzoate), in contrast to that by p-chloromercuribenzoate, was only partially reversed by dialysis against dithiothreitol.

TABLE I

Inhibition of saccharopine dehydrogenase by sulfhydryl-directed reagents

To the enzyme (4.4 nmol) in 80 μl of 0.1 M potassium phosphate buffer, pH 6.8, were added 20 μl of neutralized solution of the reagent to give the concentration indicated. The enzymic activity was determined on 5-μl aliquots at time indicated. The residual activity represents the percentage of activity obtained by comparison with the uninhibited control.

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Concentration</th>
<th>Incubation time</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mM</td>
<td>min</td>
<td>%</td>
</tr>
<tr>
<td>p-Chloromercurbenzoate</td>
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<td>0</td>
</tr>
<tr>
<td>Mersalyl acid</td>
<td>0.2</td>
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<td>0</td>
</tr>
<tr>
<td>5,5′-Dithiobis(2-nitrobenzoate)</td>
<td>0.2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>o-Iodosobenzoate</td>
<td>1.0</td>
<td>5</td>
<td>10</td>
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<tr>
<td>N-Butylmaleimide</td>
<td>0.2</td>
<td>5</td>
<td>3</td>
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<tr>
<td>N-Ethylmaleimide</td>
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<tr>
<td>Iodoacetamide</td>
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<td>45</td>
<td>36</td>
</tr>
<tr>
<td>Iodoacetate</td>
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<td>45</td>
<td>77</td>
</tr>
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</table>

Fig. 1 (left). Titration of saccharopine dehydrogenase with p-chloromercuribenzoate (PCMB). The enzyme (7.5 nmol) in 2.0 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.45 M (NH₄)₂SO₄, was incubated with p-chloromercuribenzoate as indicated. The reference cuvette contained the same reaction mixture except the enzyme. Enzymic activity was determined on 2-μl aliquots. The concentration of p-chloromercuribenzoate was estimated from its molar extinction coefficient ε₂₅₄ = 1.74 × 10⁴ M⁻¹ cm⁻¹ in 0.33 M acetic buffer, pH 4.6 (8). O, enzymic activity; O, Δ absorbance at 250 nm.

Fig. 2 (right). Titration of saccharopine dehydrogenase with 5,5′-dithiobis(2-nitrobenzoate) (DTNB). The enzyme (7.6 nmol) in 2.0 ml of 0.1 M Tris/HCl buffer, pH 8.5, containing 0.45 M (NH₄)₂SO₄ and 0.1 mM EDTA was incubated with 5,5′-dithiobis(2-nitrobenzoate) as indicated. Enzyme was omitted from the reference cuvettes. At 20-min intervals, the increase in absorbance at 412 nm was recorded, and the enzymic activity was determined on 2-μl aliquots. The concentration of 5-thio-2-nitrobenzoate released was determined from its molar absorption coefficient ε₄₁₂ = 13,900 M⁻¹ cm⁻¹ (9). O, enzymic activity; O, Δ absorbance at 412 nm.

Less than 20% of the activity was restored by dialysis.

Among three sulfhydryl groups, the difference in reactivity toward the reagent was observed. As shown in Fig. 3, the addition of 1 eq of 5,5′-dithiobis(2-nitrobenzoate) to the native enzyme caused a rapid increase, complete within 10 min, in absorbance at 412 nm. A much slower rate, comparable to that obtained with the sodium dodecyl sulfate-treated enzyme, was seen following the addition of the 2nd and 3rd eq. A total of three sulfhydryl groups were titrated with the native and denatured enzymes or when the native enzyme was treated with an excess amount of the reagent (Fig. 3, broken line).

An interesting point in this experiment is that the change in absorbance at 412 nm following the addition of a 1st and 2nd eq of the reagent exceeds that expected from the molar absorbance of thionitrobenzoate (Fig. 3, right ordinate). This anomaly could be due to an intra- or intermolecular disulfide interchange reaction between protein-S-thionitrobenzoate and protein-SH, and may reflect a poor recovery of enzymic activity after dialysis against a thiol. Similar observations are reported for phosphorylase b (10), lactate dehydrogenase (11), transglutaminase (12), α-amylase (13), pyruvate kinase (14), and so forth (see also Ref. 15).

Reaction with Iodoacetate

Previously we have demonstrated that saccharopine dehydrogenase is irreversibly inactivated by incubation with excess iodoacetate (1). The mechanism of inactivation and the identity of the residue modified by the reagent was studied in more detail.

Time Course of Inactivation—The inhibition of saccharopine dehydrogenase activity with respect to time is shown in Fig. 4. The inhibition followed pseudo-first order kinetics. In 0.1 M Tris/HCl buffer, pH 8.5, an apparent first order rate constant (kₐₙ₃) of 0.1 min⁻¹ was obtained. When kₐₙ₃ values obtained at several concentrations of iodoacetate were plotted against its concentrations, a linear relationship was obtained,
Effect of Coenzymes, Substrates, and Their Analogs on Inactivation by Iodoacetate—A previous report (1) has shown that the inhibition by iodoacetate is prevented by an excess of coenzymes, suggesting that the modified residue is located at or near the active site. The protecting effects, expressed as half-time of inactivation, of coenzymes, substrates, and their analogs are summarized in Table II. When added singly, NADH was most effective. NADPH, which is a poor coenzyme for the dehydrogenase (2), exerted a protective effect at much higher concentrations. AMP, ADP, and ATP, competitive inhibitors of NAD(H) (2) also gave protection. α-Ketoglutarate or lysine alone were virtually without effect, but their presence markedly enhanced the effectiveness of NADH. The kinetic mechanism of saccharopine dehydrogenase is a sequential ordered mechanism with coenzyme binding first and leaving last. In the direction of reductive condensation of lysine and α-ketoglutarate, the second substrate adding is O-ketoglutarate (16). In this mechanism, α-ketoglutarate, lysine, or leucine (a competitive inhibitor of lysine (17)) alone would not be able to afford a protecting effect even when the modification occurs at their binding sites. The enhanced effectiveness of NADH in the presence of α-ketoglutarate and a-

TABLE II

<table>
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<tr>
<th>Compounds added (mM)</th>
<th>t½ (min)</th>
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<tr>
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<tr>
<td>Lys (10)</td>
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<tr>
<td>α-Kg (10)</td>
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<tr>
<td>α-Kg (10) + Leu (2.5)</td>
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<tr>
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<tr>
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<tr>
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<tr>
<td>NADP⁺ (3)</td>
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<td>Saccharopine (5)</td>
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<td>ADP (2)</td>
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<td>ATP (2)</td>
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<td>ADP (2) + α-Kg (10)</td>
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Dissociation constants for NADH, a-ketoglutarate, and leucine from E·NADH, E·NADH-a-ketoglutarate, and E·NADH-a-ketoglutarate-leucine complexes as determined from their protective effects on iodoacetate inhibition

The enzyme (0.85 mU) was incubated with 400 nmol of iodoacetate in 0.1 M Tris/HCl buffer, pH 8.5, in the presence of compounds indicated. The apparent first order rate constants for inactivation (k') were determined from pseudo-first order kinetic plots. Dissociation constants were calculated by Equations 3 and 4 (see text).

\[
\text{Table III}
\]

<table>
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<tr>
<th>Compounds added</th>
<th>NADH</th>
<th>a-Ketoglutarate</th>
<th>Leucine</th>
<th>Apparent rate constant (k')</th>
<th>Dissociation constant (K')</th>
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<td>0</td>
<td>0</td>
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<td>0.55^\text{b}</td>
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<td>2.5</td>
<td>0</td>
<td>0.072</td>
<td>-</td>
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</table>

^\text{a} Calculated by Equation 3.
^\text{b} Calculated by Equation 4, with \( K_a = 0.025 \) mM.
^\text{c} Calculated by Equation 4, with \( K_a = 0.026 \) mM, \( K_b = 0.50 \) mM.

Fig. 6. Stoichiometry between loss in enzymic activity and [2-\text{\textsuperscript{14}}C]iodoacetate incorporated into enzyme. Experimental conditions are described under "Experimental Procedures." treatment. Although ADP is a good protector against the inactivation, the simultaneous addition of a-ketoglutarate brought about no enhancement (Table II, Lines 15 and 17). This is consistent with the result of kinetic analysis that the binding of ADP to the free enzyme does not provide the binding site for a-ketoglutarate (2).

Incorporation of [\text{\textsuperscript{14}}C]Iodoacetate into the Enzyme—The stoichiometry between loss in enzyme activity and the amount of iodoacetate incorporated into the enzyme was examined. Saccharopine dehydrogenase was incubated with a 10-fold molar excess of [2-\text{\textsuperscript{14}}C]Iodoacetate at pH 9.3, and, at appropriate times, aliquots were removed for measurements of enzyme activity, and radioactivity fixed. Plots of residual enzyme activity against moles of iodoacetate/mol of enzyme, indicating that the loss in activity is due to the modification of a single amino acid residue (Fig. 6).

Identification of Amino Acid Residue Modified by Iodoacetate—The amino acid analysis of saccharopine dehydrogenase that had been 96% inhibited by the reaction with iodoacetate revealed a new peak absent in the untreated enzyme. The peak, approximately 1 mol/mol of enzyme in amount, was eluted just before aspartic acid at the position expected for S-carboxymethylcysteine (6). No peak corresponding to carboxymethylhistidine or carboxymethyllysine was observed. A separate experiment showed that the iodoacetate-treated enzyme had two rather than three sulfhydryl groups titratable with 5,5'-dithiobis(2-nitrobenzoate). Moreover, an enzyme preparation that had been inhibited 70% by incubation with a small amount of p-chloromercuribenzoate showed a fixation of the label to be 30% of that of the control on further incubation with the radioactive iodoacetate. These results establish the identity of the modified residue as cysteine residue, and indicate that the same cysteine residue is modified by iodoacetate and p-chloromercuribenzoate.

Equilibrium Dialysis Studies—The binding of NADH to the native and carboxymethylated enzyme was measured by equilibrium dialysis. The binding to the native enzyme showed a saturation curve as depicted in Fig. 7. A Scatchard plot (19) shown in the inset gave a value of 50.5 \mu mol for the dissociation constant with one binding site/mol of enzyme. The alkylated enzyme, on the other hand, was devoid of binding capacity. Similar binding experiments with radioactive a-ketoglutarate
and leucine showed that they are bound to the enzyme only in the presence of NADH in the former, and NADH plus α-
ketoglutarate in the latter, confirming the ordered binding of
reactants predicted by kinetic analyses.

**Reaction with N-Ethylmaleimide and Iodoacetamide**

The inhibitory effect of N-ethylmaleimide and iodoacetamide
was studied under the same conditions as for iodoacetate.
The reaction was second order in both cases. The second
order rate constants for N-ethylmaleimide and iodoacetamide
were 2.6 mm⁻¹ min⁻¹ and 1.2 mm⁻¹ min⁻¹, respectively. These
values are an order of magnitude greater than that for iodoac-
etate.

**DISCUSSION**

The yeast saccharopine dehydrogenase is a monomeric
enzyme and contains only 3 cysteine residues/mol. Thus,
among dehydrogenases, the enzyme may be expected to pro-
vide a useful system for studying the functional role played
by the sulphydryl group. In fact, saccharopine dehydrogenase
inhibited by a variety of sulphydryl reagents (Table I). The
reaction of the enzyme with iodoacetate results in the forma-
tion of 1 mol of S-carboxymethylcysteine/mol of enzyme. A
pKᵦ value of 9.3 for inactivation and a loss of one titratable
sulphydryl group are also consistent with the fact that the site
of modification is a cysteine residue. The 1:1 stoichiometry
between the loss in enzymic activity and the amount of residue
modified seen with p-chloromercuribenzoate, 5,5'-dithiobis(2-
nitrobenzoate), and iodoacetate as inhibitors indicates that
one very reactive thiol group is directly involved in catalytic
activity of the enzyme. The experiment shows that the enzyme
preparation that has been partly inhibited by the reaction
with a fractional molar concentration of p-chloromercuriben-
zoate is alkylated by iodoacetate only to an extent correspond-
ing to the residual activity and thus indicates that the same
thiol group is being blocked by these reagents. Our previous
report (1) that the reaction of three sulphydryl groups with p-
chloromercuribenzoate is necessary for complete loss in cata-
lytic activity could not be confirmed in the present study. The
previous conclusion appears to be incorrect, because under
the conditions employed it was found to be difficult to deter-
mine the termination of reaction of p-chloromercuribenzoate
with the enzyme. This was also the case for the reaction with
5,5'-dithiobis(2-nitrobenzoate). The difficulty could be cir-
cumvented by the addition of relatively high concentrations
of (NH₄)₂SO₄. The increase in reactivity of sulphydryl groups
toward p-chloromercuribenzoate and 5,5'-dithiobis(2-nitro-
benzoate) in the presence of salts such as (NH₄)₂SO₄, pyro-
phosphate, or perchlorate is reported (15), although the mech-
anism of activation is not fully understood.

Saccharopine dehydrogenase is protected from inactivation
by iodoacetate in the presence of the coenzyme and its frag-
ments, AMP, ADP, and ATP. The effectiveness of these
compounds closely parallels their affinities for the enzyme as
indicated by their Kᵦ values (2, 17, 18). α-Ketoglutarate, lysine,
or leucine (a competitive inhibitor of lysine (17)), alone or in
combination, was ineffective as expected from the ordered
nature of reaction, but when they are added together with
NADH, the protective effect of the latter is greatly enhanced
(Table II). Calculation of dissociation constants for the coen-
zyme and substrate (or its analog) on the assumption that the
reaction of iodoacetate takes place only with the free enzyme
(Equation 2) gives values very close to those obtained kine-
tically (Table III). These results strongly support the idea that
the modification occurs on a cysteine residue located near the
binding site for the adenosine moiety of the coenzyme. The
reactions of p-chloromercuribenzoate and 5,5'-dithiobis(2-ni-
trobenzoate), however, were not prevented by the coenzyme
in spite of the fact that apparently the same sulphydryl group
is blocked by these reagents. The reason for this is not clear
at present.

As shown by an equilibrium dialysis study, the carboxy-
methylation of saccharopine dehydrogenase appears to result
in the loss in ability to bind the coenzyme. Kinetic analysis
with a partially inhibited enzyme showed that the modifica-
tion alters only Vᵦ while the maximum values for the enzyme
(data not shown). This is in agreement with the inability of the enzyme
to bind reactants, although other explanations may be possi-
ble.

The observations are consistent with any of the following
roles for the sulphydryl group. (a) It directly participates in
coenzyme binding; (b) the modification alters the enzyme
conformation required for coenzyme binding; or (c) by modi-
fication the coenzyme becomes inaccessible to the binding site
for steric and other reasons although no gross conformational
change occurs. In any event, the mechanism of inhibition of
saccharopine dehydrogenase by sulphydryl reagents appears
to be different from that for liver (20, 21) and yeast (22, 23)
alcohol dehydrogenases, and lactate dehydrogenase (24, 25).
In these enzymes, it is reported that the modification of
"essential" thiol groups does not abolish their ability to bind
a coenzyme.

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**Fig. 7.** Equilibrium dialysis of the native and carboxymethylated
enzymes. The experimental conditions and procedure are described
under "Experimental Procedures." For the preparation of carboxy-
methylated enzyme, saccharopine dehydrogenase was treated with
20-fold molar excess of iodoacetate in 0.1 M Tris/HCl buffer, pH 9.3.
When 95% of initial activity was lost, an excess amount of dithiothre-
titol was added. After dialysis overnight against 0.1 M Tris/HCl buffer,
the protein concentration was adjusted to 12.8 μM. Inset shows a Scatchard plot
for the native enzyme. 1 represents the number of moles of NADH bound/mol enzyme, and f the concentration of free NADH.

The line was drawn by a least squares linear regression.
Sulfhydryl Groups of Saccharopine Dehydrogenase


Chemical modification of the active site sulfhydryl group of saccharopine dehydrogenase (L-lysine-forming).
H Ogawa, M Okamoto and M Fujioka


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