Vanadate was used as a substrate analogue to modify and subsequently localize active site serine residues of isocitrate lyase from *Escherichia coli*. Irradiation of the enzyme on ice with UV light in the presence of vanadate resulted in inactivation. Inactivation was prevented by the substrates glyoxylate or D, isocitrate and to a much lesser extent by succinate. Reduction of photoinactivated isocitrate lyase by NaBH₄ partially restored enzyme activity. The photomodified enzyme was labeled by reduction with NaB[3H]₄ in the presence and absence of the substrates succinate plus glyoxylate. Highly differential labeling of serine residues 319 and 321 in the absence of substrates suggests their importance in the action of isocitrate lyase. These residues are highly conserved in all five known sequences of this enzyme.

**RESULTS**

**Photoinactivation of Isocitrate Lyase in the Presence of V₅**—Isocitrate lyase was inactivated by UV irradiation in the presence of V₅, whereas in the absence of V₅, the enzyme remained active during 30 min of irradiation.
The enzyme was also stable for 2 h at 4 °C in the dark in the presence of 0.4 mM V, (data not shown). A combination of substrates, glyoxylate and succinate, each at 10 mM, provided substantial protection against photoinactivation (Fig. 1). The substrate concentrations were well above the K_0 and K_v values of 0.28 and 0.45 mM, for glyoxylate and succinate, respectively (Ko and McFadden, 1990b). The level of protection was greatest (75%) after 10 min irradiation, diminishing to 50% after a further 20 min of irradiation.

The protection by substrates against V, photoinactivation of isocitrate lyase (Fig. 1), indicates that the modification occurred at the active site of the enzyme. However, this effect could also be explained by a photoinduced oxidation of the substrates with a concomitant reduction of V, to a lower oxidation state. This diminished concentration of the +5 oxidation state of vanadium would decrease the rate of the protein oxidation reaction. A reaction between Tris and V, has been shown to occur in studies on the photocleavage of myosin (Cremo et al., 1990). To test for a reaction of V, with glyoxylate (the most likely substrate to be oxidized), we irradiated 10–200 μM glyoxylate with 0.2 mM V, in MME buffer for 30 min. Our sensitive colorimetric assay for glyoxylate (Jameel et al., 1984) could not detect a decrease in glyoxylate concentration under these conditions. Thus, the apparent protection by substrates seen in Fig. 1 was not due to a significant decrease in the concentration of V, or substrates but is likely indicative of active site modification by V,.

To further examine the specificity of the V,–mediated photoinactivation, samples of isocitrate lyase containing 0.4 mM V, were irradiated for 10 min in the presence and absence of various substrates (Fig. 2). In the absence of substrates, less than 10% of the control activity remained. Ten mM Ds-isocitrate (K_0, 0.14 mM; Ko and McFadden, 1990b) or glyoxylate (K_0, 0.28 m molt) protected substantially against the V,–dependent inactivation, whereas succinate provided the least protection. No significant additional protection was afforded by the combination of glyoxylate and succinate.

**Restoration of Activity by Reduction with NaBH_4.**—It has been previously shown (Cremo et al., 1988; Mogel and McFadden, 1989) that oxidized serine residues (serine aldehydes) in proteins can be reduced back to serine with NaBH_4. If V, was oxidizing serine residues to the aldehyde form at the active site of isocitrate lyase, treatment with NaBH_4 should restore enzyme activity. To test for this possibility, photoinactivated isocitrate lyase (by 0.4 mM V, for 10 min) and an unirradiated control sample were treated with varying concentrations of NaBH_4 for 1 h (Fig. 3). Enzyme activity was restored from 16% (without reduction) to 52% (with 13.3 mM NaBH_4) relative to a control sample that was treated with V, but not irradiated. Under these conditions, a glyoxylate-protected sample (see Fig. 2) retains approximately 75% of the control activity. Thus the maximal NaBH_4 treatment of the V, light-inactivated sample (Fig. 3) restored 52/75 or 70% of the expected activity. These data showing appreciable reversal of the inactivation by V, indicate that the photooxidation reaction involves serine residues at the active site of isocitrate lyase.

**Photocleavage.**—V, oxidations of proteins induced by UV light often result in cleavage of the polypeptide chain (see reviews in Cremo et al., 1991; Gibbons and Moc, 1991). Irradiation of isocitrate lyase, known to consist of four 47.2-kDa subunits (Matsuoko and McFadden, 1988), in the presence of 0.4 mM V, as for Fig. 1 generated one predominant new polypeptide, as evidenced by SDS-PAGE, migrating at an apparent molecular mass of 36–37 kDa and a minor peptide of 10–12 kDa. The photocleavage in MME buffer (3.3 mM MOPS) to form the larger peptide was approximately 4% of the isocitrate lyase after 30 min of irradiation (Fig. 4A) and was prevented by 10 mM glyoxylate plus 10 mM succinate (Fig. 4B). Neither NH_2-terminal analysis or sequencing of the...
carboxyl-terminal side of the cleavage site was attempted, as
it has been previously shown for myosin (Mocz, 1989; Cremo et al., 1991) and dynein (Gibbons and Mocz, 1991) that these termini are blocked.

HPLC of Tryptic Digests and Sequencing of the Labeled Peptides—As the V<sub>i</sub> inactivation of isocitrate lyase appeared to be specific for the active site (Figs. 1 and 2) and appeared to involve serine residues (Fig. 3), the identification of serines that are specifically oxidized was undertaken by characterization of <sup>3</sup>H-seryl residues after NaBH₄ treatment. Fractions from a tryptic digestion of isocitrate lyase that had been irradiated in the presence of V<sub>i</sub> (with and without substrates) and subsequently reduced with NaBH₄, were separated by HPLC (Fig. 5). The level of radioactivity incorporated into four peaks (labeled 1–4, <i>inset</i>) was diminished by 50–65% (see <i>inset</i>, Fig. 5) in the presence of the substrates glyoxylate and succinate (each at 5.7 mM) during the irradiation. The sequencing of peptides in peaks 1 and 2 revealed little or no radioactivity in any of the cleavage cycles, and no radioactivity remaining on the sequencing filter. Apparently, the radioactivity observed in these peptides (Fig. 4) was lost under the strongly acidic sequencing conditions. In contrast, in sequencing both peaks 3 and 4, most of the radioactivity was present in the ninth cycle which corresponds to serine 321 in the sequence LLAYNCSPSFNWQK (Fig. 6). Radioactivity started to appear in the seventh cycle corresponding to serine 319. Serine 319 appeared to be labeled to a much lower degree than serine 321, as the yield for cycle 7 was 31 pmol and for cycle 9 was 21 pmol. Presumably, the occurrence of an identical peptide, LLAYNCSPSFNWQK, in both peaks (Fig. 6) reflected the separation of an ion pair (or another tight intramolecular complex) in either peak 3 or 4. Indeed both peaks 3 and 4 each contained one additional minor peptide which did not contain serine. Sequences of these minor peptides were: AMIEAGAAAVHFED for peak 3 and GLAYAPYADLVWCE for peak 4, corresponding to positions 174–187 and 276–289, respectively. It is assumed that these peptides did not contain radiolabel, as the amino acids in position 7 and 9 (A-A-Y-D) could not be oxidized and subsequently reduced with NaBH₄ to produce unmodified <sup>3</sup>H-amino acids.

DISCUSSION

Isocitrate lyase from E. coli is photoactivated by UV irradiation in the presence of V<sub>i</sub>. In the dark in the presence of V<sub>i</sub> or in the light in the absence of V<sub>i</sub> (Fig. 1) there is no apparent inactivation of the enzyme. The patterns of protection afforded by the substrates of isocitrate lyase against photoactivation (Figs. 1 and 2) suggest that photooxidation occurs at the active site. For example, the observation that succinate provides considerably less protection than glyoxylate or isocitrate is consistent with the known ordered steady-state bi-uni kinetic mechanism in which succinate does not bind productively to free enzyme but only to the enzyme-glyoxylate complex (Ko and McFadden, 1990b). Moreover, it is consistent with the protection patterns observed during covalent modification in the active site domain of this enzyme by diethylpyrocarbonate (Ko et al., 1991) and bromopyruvate (Ko and McFadden, 1990a), both of which are affinity labels.

The restoration of enzyme activity by treatment with NaBH₄ suggests that a derivative of isocitrate lyase containing a reactive carbonyl is generated during the photoactivation. In studies on the photomodifications of myosin in the presence of V<sub>i</sub>, a serine residue is oxidized to the corresponding aldehyde, which can be reduced back to serine by treatment with NaBH₄ (Cremer et al., 1988). In the present investigation, the activity of photoactivated isocitrate lyase could be returned to approximately 70% of control levels by treatment with NaBH₄. Less than full recovery of activity after reduction suggests that some of the oxidized serine residues have proceeded past the aldehyde level of oxidation, to generate moieties that do not react with NaBH₄. Evidence for intermediates between initial oxidation and photocleavage has been documented in the V<sub>i</sub>-mediated reactions with adenylate kinase.<sup>2</sup>

The above results are consistent with the observation that some isocitrate lyase is photocleaved under our irradiation conditions. The cleavage may be in the active site domain because it is abolished by the substrates glyoxylate and succinate. Furthermore, the position of the cleavage site appears to correspond to the positions of serine oxidation described below. It is not known why the extent of photocleavage is so small (4%). In the case of myosin, further oxidation of the active site serine 180 aldehyde leads to nearly quantitative cleavage of the protein in skeletal myosin (Grammer et al., 1988), but the same treatment with smooth muscle myosin (Cole and Yount, 1991) results in <10% photocleavage.<sup>3</sup> The sequences of the two protein forms are identical in the vicinity of serine 180.

The incorporation of tritium by treatment of photooxidized isocitrate lyase with NaBH₄ appeared to be highly specific. Only four substrate-protectable radioactive peaks in an HPLC chromatogram were identified after complete trypsinolysis of the enzyme (Fig. 5, <i>inset</i>). As expected from the inactivation studies (Figs. 1 and 2), the substrates glyoxylate plus succinate afford only partial protection as measured by radioactivity incorporated into the peptides. Serine 321 appeared to be the predominantly labeled residue with serine 319 labeled to a lesser extent. In this connection, it is of interest that the sizes of the fragments arising from photocleavage are consistent with cleavage in the region of these serine residues. Serine 319 and 321 are presumably functionally important because

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<sup>2</sup> C. Cremer, unpublished observations.

<sup>3</sup> D. Cole, unpublished observations.
Photoinactivation of Isocitrate Lyase by Vanadate

Fig. 5. HPLC separation of tryptic peptides of reduced isocitrate lyase. Isocitrate lyase (45 μM subunits) in MME buffer (50 mM MOPS) containing 0.4 mM V, was irradiated for 30 min without and with glyoxylate plus succinate (each at 5.7 mM). After addition of triethanolamine to 81 mM, the enzyme was subsequently reduced for 2.5 h in the dark on ice with NaB3H4 (76 μCi of tritium) resulting in a total volume of 2.5 ml. To denature and precipitate the enzyme, 4 ml of absolute ethanol was added and the mixture incubated on ice for 30 min and centrifuged (Sorvall SS-34 rotor at 10,000 rpm) for 30 min; the pellet was washed exhaustively with cold absolute ethanol and dissolved in 50 mM MOPS (pH 8.3). Trypsin was added in a mass ratio of 1:100. After 12-h digestion at 37 ºC, the samples were passed through a 0.22-micron filter before subjecting them to HPLC using a Brownlee Lab Reverse Phase C8 column (30 X 4.6 cm) under the following operating conditions: flow rate, 1 ml/min; eluting solvent A: deionized water containing 0.1% trifluoroacetic acid; eluting solvent B: 70% acetonitrile containing 0.1% trifluoroacetic acid; linear gradient, 0-100% B during 50 min; fraction size, 1 ml. Thirty μl of each collected fraction was used to determine the radioactivity shown in the inset (plus glyoxylate and succinate, each at 5.7 mM (0); no substrates (0)). The numbers 1-4 indicate the positions of the specifically labeled peptides. The large peak of radioactivity eluting at 4-6 min (void volume) corresponds to tritium that was not covalently incorporated into the protein. Fractions 46-75 (not shown) had background levels of radioactivity.

Fig. 6. Distribution of radioactivity for each amino acid residue obtained in sequencing radioactive peaks 3 and 4 as isolated by HPLC (see Fig. 5). The sequence of both serine-containing tryptic peptides is shown and was identical in both peaks. Sequencing was done with an ABI 12-A gas-phase sequenator (Applied Biosystems, Inc.) and an ABI 12-A phenylthiohydantoin analyzer. Data acquisition and analyses were performed with the standard program RUN470-1 (ABI).

The sequences of the amino acid residues surrounding them are highly conserved in all known aligned primary structures for isocitrate lyase (Table I). Hence serine 321 (and secondarily serine 319) in E. coli isocitrate lyase and counterparts in this enzyme from other sources may be functional, presumably in the binding of isocitrate or glyoxylate. The placement of these serine residues in the active site domain of isocitrate lyase augments our placement of cysteine 195, also a conserved residue (Matsuoka and McFadden, 1988), in the same domain (Ko and McFadden, 1990a).

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REFERENCES


TABLE I

<table>
<thead>
<tr>
<th>Source</th>
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<tr>
<td>Rape seed3</td>
<td>M-L-A-Y-N-L-S-P-S-F-N-W-D-A</td>
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<tr>
<td>Yeast6</td>
<td>L-L-A-Y-N-C-S-P-S-F-N-W-Q-K</td>
<td>313-326</td>
</tr>
</tbody>
</table>

3 Comai et al. (1989).
5 Turley et al. (1990).
6 Atomi et al. (1990).
8 Matsuoka and McFadden (1988). Serine 319 and serine 321 are underlined in the E. coli tryptic peptide.