Dehydroquinase, the third enzyme of the shikimate biosynthetic pathway, is inactivated by iodoacetate. Iodoacetate behaves as an affinity label for the *Escherichia coli* enzyme with a *K*ᵢ of 30 mM and a limiting inactivation rate of 0.014 min⁻¹ at pH 7.0 and 25 °C. Affinity labeling is mediated by the negative charge of the reagent since iodoacetamide does not inactivate the enzyme. 2.1–2.3 mol of carboxymethyl groups are incorporated per mol of protein monomer resulting in 90% inactivation of enzymic activity. The majority of the bound label (80%) is split equally between 2 methionine residues, Met-23 and Met-205, which were identified by sequencing radiolabeled peptide fragments isolated after proteolytic digestion. An equilibrium mixture of the substrate (dehydroquinate) and product (dehydroshikimate) substantially reduces the inactivation rate and specifically decreases the incorporation of label at both of these sites, implicating them as being in or near the active site of the enzyme. Sequence alignments with other biosynthetic dehydroquinases show that of the 2 methionine residues only Met-205 is conserved. N-terminal alignments of all the available dehydroquinase sequences (both catabolic and biosynthetic classes) revealed that Met-23, although itself not conserved, resides within a cluster of conserved sequence which may constitute part of the dehydroquinase active site binding site. A consensus sequence was derived from these alignments and used to probe the protein sequence data banks. A related sequence was found in dehydroquinase synthase, the enzyme which precedes dehydroquinase in the shikimate pathway. These results suggest that we have identified part of the dehydroquinate binding site in both enzymes.

The biosynthetic class of dehydroquinase enzymes consists of three known, constitutively expressed subtypes: a monofunctional, of which the *Escherichia coli* enzyme has been the most widely studied (Berlyn and Giles, 1969; Chaudhuri et al., 1987), and which has been purified to homogeneity (Chaudhuri et al., 1987), and its gene (aroD) has been cloned, sequenced, and overexpressed (Duncan et al., 1986); a bifunctional form found in plants, which is partnered by another shikimate pathway enzyme, shikimate dehydrogenase, and which has been purified from a moss and two legume species (Polley, 1978; Koshiha, 1978; Mousdale et al., 1987); and finally a multifunctional form in which the dehydroquinase activity is located on one domain of a large pentafucntional polypeptide, the *aroM* multifunctional enzyme. This multifunctional enzyme, which possesses the enzymatic activities for the five central steps of the shikimate pathway, has been purified to homogeneity from *Neurospora crassa* (Lumsden and Coggins, 1977; Gaertner and Cole, 1977; Coggins et al., 1987). The gene encoding the *aroM* multifunctional enzyme has been cloned and sequenced from *Aspergillus nidulans* and Saccharomyces cerevisiae (Charles et al., 1985, 1986; Duncan et al., 1987). The catabolic dehydroquinases, which are monofunctional, are found in fungi where they, and the rest of the enzymes of the quinate pathway, are inducibly expressed. The catabolic dehydroquinases of both *N. crassa* and *A. nidulans* have been purified (Hautala et al., 1975; Hawkins et al., 1982) and their genes cloned and sequenced (Da Silva et al., 1986; Geever et al., 1989).

The two classes of dehydroquinase are markedly different from each other in three respects: 1) the molecular mass for the biosynthetic polyepitope is ~27,500 daltons whereas the catabolic polyepitope is ~17,000 daltons; 2) the proteins within a class are homologous to each other but little or no sequence homology has been observed between each class; and 3) the catalytic and physical properties of the *E. coli* biosynthetic enzyme are dramatically different to those of the fungal catabolic enzymes. For example, the bacterial enzyme is dimeric, has a *Kₗ₉* for 3-dehydroquinic acid of 18 μM (similar to that of the dehydroquinase component of the *aroM* multifunctional protein), and is heat-labile, whereas the fungal enzymes are dodecameric, have a *Kₐ₉* for 3-dehydroquinic acid of ~500 μM, and are very stable at elevated temperatures (as described by White et al., 1990).

The stereochemical course of this reaction has generated some interest because it proceeds by a syn elimination (Hanson and Rose, 1963), which contrasts the trans stereochemistry more commonly observed for such elimination reactions (Haslam, 1974). Borohydride inhibition of enzyme in the presence of substrate has shown that a Schiff base is involved...
in the mechanism (Butler et al., 1974), and this may help to explain the preference for syn elimination on the surface of the enzyme (Vaz et al., 1975). The site of Schiff base formation has been identified both in the E. coli enzyme (Lys-170) and in the dehydroquinase component of the N. crassa multifunctional enzyme, which corresponds to Lys-1227 in the published sequence of S. cerevisiae (Duncan et al., 1987). A general base has been implicated in the mechanism (Chaudhuri et al., 1986), but this has not been identified. Toward this end, substrate-based affinity labels have been synthesized (Bugg et al., 1988) but these have yet to be characterized fully.

The aim of this work was to identify active site nucleophiles in the E. coli biosynthetic dehydroquinase using the electrophilic reagent iodoacetic acid. Iodoacetate is an effective, active-site directed inhibitor of the E. coli enzyme; the sites responsible for inactivation have been identified as 2 methionine residues, one near the N terminus and the other near the C terminus of the protein. Modification of the methionine residue near the N terminus has highlighted a cluster of previously unrecognized, conserved sequence which is present in both the biosynthetic and catabolic dehydroquinases and may represent part of the binding site for 3-dehydroquinic acid in the two classes of enzyme. Surprisingly, a similar sequence is also present in dehydroquinase synthase, the enzyme which precedes dehydroquinase in the shikimate pathway. This is the first report of a common structural motif found within the enzymes of the shikimate pathway.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

Iodoacetic acid behaves as an affinity label for E. coli dehydroquinase. The inactivation kinetics exhibited by this simple reagent conform to the classic definition of an affinity label (Shaw, 1970; Pfapp, 1982), in that the rate of inactivation saturates as the reagent concentration is increased, and the inactivation rate is reduced by a mixture of substrate and product. Saturation of the inactivation rate shows that a specific Michaelis complex is formed with the enzyme prior to inactivation (Kitz and Wilson, 1962; Meloche, 1967), and a dissociation constant for iodoacetic acid of 30 mM is obtained from these data. This is similar to that for acetic acid (102 mM, Chaudhuri et al., 1986), a competitive inhibitor of the enzyme. Affinity labelling by iodoacetic acid is mediated by the negative charge of the reagent since iodoacetamide does not inhibit the enzyme. Similar differential reactivities for these two reagents have been reported for ribonuclease T1 (Takahashi et al., 1967), an observation consistent with an anionic binding site on the enzyme attracting the carboxylate of the reagent. Since both iodoacetate and dehydroquinase are negatively charged it seems likely that an anionic binding site exists within the substrate binding pocket of dehydroquinase.

The stoichiometry of labeling dehydroquinase by iodoacetic acid indicates that 90% loss of activity is accompanied by the incorporation of approximately 2 mol of carboxymethyl groups. The majority of the label (80%) was distributed equally between two tryptic fragments, TP1 and TP2. The remaining 20% of incorporated label was spread over a number of different elution positions in the peptide map and these were not investigated further. When the inactivation was protected by the inclusion of the substrate/product equilbrium mixture the incorporation of label at TP1 and TP2 was significantly reduced; this suggests that both peptides are derived from the active site region of the enzyme and that the alkylation of both sites results in the loss of enzymatic activity.

TP1 and a chymotryptic subfragment of TP2 were purified to homogeneity, and the position of label was determined. In both cases, the site of carboxymethylation was found to be a methionine residue. TP1 was identified as an 8-residue tryptic fragment originating from the N-terminal region of dehydroquinase (residues 18-25) and the modified residue was identified as Met-23. The peptide TP2/CT was found to be a 4-residue fragment derived from the C-terminal third of the enzyme (residues 204-207), and the modified residue was Met-205. There is no preferential protection of one site over the other, as the incorporation of label is reduced equally at both methionine residues by the substrate/product mixture. Moreover, when the incorporation of label into TP1 and TP2 was monitored during inactivation of the enzyme, both sites were labeled simultaneously, discounting the possibility that one site is modified before the other (data not shown).

The modification of methionines by a-halogenocarbonyl reagents is rare by comparison with the modification of more nucleophilic side chains such as those of cysteine and histidine. Although dehydroquinase was originally thought to contain cysteine residues (Duncan et al., 1986) sequencing errors have recently been identified which remove all 3 cysteines: one at position 99 (which is now alanine in the amended sequence) and a frame shift in the extreme C terminus which not only lengthens the sequence by 12 residues but also deletes the remaining 2 cysteine residues. The regions of sequence presented in this study are unaffected by these amendments. The corrected sequence will be published elsewhere. The next mostreactive groups are histidine residues, but no carboxymethyl histidine residues were found in the modified protein either by amino acid analysis or by high voltage electrophoresis (data not shown).

Since methionines are seldom implicated as catalytic residues it was of interest to determine whether Met-23 and Met-205 were conserved in other biosynthetic dehydroquinase sequences. Sequence comparisons with two homologous dehydroquinases, the dehydroquinase domains within the polyfunctional protein of S. cerevisiae and A. nidulans, reveals that, of the two methionine residues, only Met-205 is conserved (Fig. 8).

The simultaneous alkylation of the two methionine residues in an active site-directed fashion is an unusual observation which, in the absence of any structural information, is difficult to interpret. A consequence of this dual alkylation is the uncertainty it generates in defining the role of each residue in either the mechanism or the structure of the enzyme. Notwithstanding these limitations some speculation is warranted, based on data presented in this and the accompanying article. Of immediate concern is whether either residue participates directly in the mechanism. We feel this is unlikely for several reasons: 1) Although alkylation of both residues is required for inactivation (the degree of modification of each residue determines the degree of residual activity) if either were a critical active site nucleophile it would be anticipated that a single alkylation would completely destroy the activity of the enzyme. 2) The label at Met-205 can be selectively removed by mercaptoethanol (described in Kleanthous and

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2 Portions of this paper (including "Experimental Procedures," "Results," and Tables I and II and Figs. 1-9) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

3 L. D. Graham, unpublished data.
Coggins, 1990) resulting in only 50% reactivation of the enzyme. The inference from this observation is that complete alkylation of Met-205 only accounts for one-half of the inactivation of the enzyme, the remaining loss of activity being attributable to the complete alkylation of Met-23. It is unlikely, therefore, that either methionine residue is critical for activity. 3) Met-23 is not conserved in the biosynthetic enzymes (Fig. 8) which further supports the notion that it is not involved in catalysis or substrate binding.

The question arises, therefore, of how alkylation of these residues inactivates the enzyme? Since they are both modified in an active site-directed fashion and are both protected by the substrate/product equilibrium mixture they are likely to reside in or near the active site of the enzyme. We propose that the observed loss of activity may simply be a consequence of introducing deleterious negative charges in the vicinity of the substrate binding pocket. Indeed, sequence alignments around Met-23 (see below) suggest that it may be part of the dehydroquinate binding site. Evidence to support this hypothesis comes from the kinetic properties of the reactivated enzyme (discussed in Kleanthous and Coggins, 1990) in which alkylation at Met-205 has been selectively removed. This protein, which is still carboxymethylated at Met-23, has an elevated \( K_{\text{m}} \) but the \( k_{\text{cat}} \) value remains unchanged.

Although methionine at position 23 is not observed in other dehydroquinase enzymes, sequence alignments show that it lies within a cluster of conserved residues in the N-terminal regions of both the biosynthetic and catabolic enzymes (Fig. 9). Modification of Met-23 has therefore highlighted a previously unrecognized element of conserved primary structure observed in both classes of dehydroquinase enzymes. We speculate that this cluster of conserved sequence may represent part of the dehydroquininate binding site in dehydroquinases and that the modification of Met-23 by iodoacetic acid has served as a “molecular tag” of this binding site. A conserved sequence was derived from these alignments and used to probe the NBRF protein sequence data banks (the probe sequence is shown in Fig. 9), with the aim of identifying similar motifs in other proteins. The search revealed that a related sequence is present in dehydroquininate synthase, the enzyme which precedes dehydroquinase in the shikimate pathway. Closer inspection of these sequence alignments highlighted two further points: 1) a conserved sequence is apparent between both classes of dehydroquinase and the synthases which differs only marginally from the probe sequence; and 2) the sequences from the \( E. \ coli \) dehydroquininate synthase and dehydroquinase show the greatest similarity to each other, with 11 of the 19 residues encompassed by the consensus sequence being of the same type. This consensus sequence was not detected in any of the other shikimate pathway enzymes.

The tentative conclusion from these alignments is that this motif is part of the dehydroquininate binding site and is common to both dehydroquininate synthase and the biosynthetic and catabolic dehydroquinases. This interpretation has some appeal since dehydroquininate is common to both the synthase and dehydroquinase, as the product of the former and substrate for the latter.

Recent work on the reaction mechanism of dehydroquininate synthase (Bartlett and Satake, 1986; Widlanski et al., 1989) suggests that the enzyme may not, in fact, bind dehydroquininate because the final chemical conversion of the enol pyranose (the postulated end-product of the synthase) to dehydroquininate proceeds spontaneously and does not require the enzyme. However, 3-deoxy-D-arabino-heptulosonic acid 7-phosphate (the substrate for dehydroquininate synthase) is thought to bind to the enzyme in a closed ring chair conformation, which is structurally very similar to dehydroquininate (Fig. 1) and the conserved motif found in dehydroquininate synthase and dehydroquinase may represent a common site for binding such structures. If this is the case then the absence of this motif in the other shikimate pathway enzymes is not surprising since they bind unsaturated substrates in which the ring can no longer adopt a chair conformation.

The structural similarity of one enzyme to the next within a metabolic pathway can be inferred in one of two ways: either from extensive homology at the amino acid level, as observed in the methionine biosynthetic pathway (Belfaiza et al., 1986; Parsot et al., 1987), or from crystallographic information which identifies common folding patterns within enzymes of a metabolic pathway, which may have dissimilar primary sequences, as observed amongst the glycolytic enzymes (Fothergill-Gilmore, 1986). The amino acid sequences for all the shikimate pathway enzymes from \( E. \ coli \) are known, and they show no overall homology. However, in the absence of any crystallographic information the question of more subtle structural homology between the enzymes of this pathway cannot be easily addressed. Identification of a common structural motif in both dehydroquininate synthase and dehydroquinase, by chemical modification, holds promise for further studies aimed at identifying substrate binding sites within other enzymes of this pathway. We are extending these studies to substrate-based reagents which are more likely to yield this information: for example, we are in the process of characterizing affinity labels for dehydroquinase (Bugg et al., 1988). This is part of the wider strategy of using chemical modification and affinity labelling to identify substrate binding sites within the enzymes of the shikimate pathway which may, in turn, lead to the identification of other common structural motifs.

Acknowledgments—We gratefully acknowledge John Greene for providing purified dehydroquinase for this study and Dr. Lloyd Graham both for the computer search of the protein sequence data banks and for helpful discussions. We also thank Dr. Tim Bugg for many stimulating and provocative discussions.

REFERENCES
Chemical Modification of Dehydroquinate


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**EXPERIMENTAL PROCEDURES**

**Materials.** Dehydroquinase acid was prepared as described by Coggins et al. (1987). Isomeric acid, 2-3-dehydroquinic acid, and 3,4-dehydroquinic acid were purchased from Sigma Chemical Co. HPLC grade trifluoroacetic acid and trifluoroacetic acid anhydride was obtained from Rathburn (Chemicals Ltd., U.K.). 2-Chloroquinolin-4-one was purchased from American Chemical Society (ACS)-grade reagent. All other chemicals were of the highest commercial grade.

**Enzyme assay and protein determination.** Dehydroquinase was purified from E. coli strain AB1654 (as previously described, Cheesbrough, B. (1977) unpublished observations) and from 3,4-dehydroquinase in E. coli strain 4134 (as previously described, Cheesbrough, B. (1977) unpublished observations).

**Purification of dehydroquinase by ion-exchange chromatography.** Dehydroquinase acid (0.04 ml) was stored in the dark at room temperature (RT) for 4 weeks and then used for the assay. On storage, the solution contained 3% 3,4-dehydroquinic acid.

**Purification of dehydroquinase by gel filtration.** The enzyme was purified as described by Cheesbrough, B. (1977) unpublished observations. The final concentration of the enzyme, which was determined according to the method of Koshita et al. (1978) Biochim. Biophys. Acta 522, 10-18.

**Identification of dehydroquinase by sodium dodecyl sulfate polyacrylamide gel electrophoresis.** The enzyme was purified as described by Cheesbrough, B. (1977) unpublished observations. The final concentration of the enzyme, which was determined according to the method of Koshita et al. (1978) Biochim. Biophys. Acta 522, 10-18.

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Chemical Modification of Dehydroquinase

Overnight digestion with chymotrypsin yielded a mixture of fragments in which one peptide containing 72% of the label was identified (TPPCT). The minor labeled peptides, which accounted for the remaining 28% of label, were not further digested at the amounts required to TPCT, or digested by amino acid analysis (data not included) they were sequenced by gas-phase sequencing and the release of radioactive Sz (Tables I & II, respectively). The sequence of both TP and TPPCT was in agreement with the recent amendment to the sequence of Bocca et al. (1984) (see diagrams). Both peptides were purified twice (two separate enzyme preparations and independent ionizations) and in each experiment the sequences and radioactivity of the labeled peptides were identical. Table I and II show the data for one of these experiments. In both peptide there is 100% reduction in yield of tyrosine in dehydroquinone in the cycle corresponding to methionine followed by methionine in the next and following cycles, suggesting that the methionine residues have been modified. In TPCT cycle corresponded exactly to the position of label. The cycle of label during the sequencing of TP and TPPCT was not quite before methionine, corresponding in a different residue. These results cannot reflect the activity of dehydroquinase and the mecano monomer is dramatically reduced. We conclude that the template monomer is the monomer that, for reasons which are unclear, much of the radioactivity stems one cycle early. amino acid analysis of each of TP and TPPCT further supports these results since each showed the presence of homoaspartic acid and homoserine, the major tautomers of amino and hydroxy amino acid oxycarboxylic acids (Bocca et al., 1984).

Probing the protein sequence database. A common sequence was derived from the N-terminal alignments of both kinetocenic and carbomethyl dehydroquinase (shown in Fig.9) and used to order the NBD protein sequence data base (Beveridge et al., 1986) for homologies in other enzymes.

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Dehydroquinate

Dehydroshikimate

3-Deoxy-D-arabino-heptulosonate 7-phosphate

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Fig. 1 Reaction catalyzed by dehydroquinase and the structure of DAH.

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Fig. 2 Incucilisation of dehydroquinase by carbomethyl (CAK).

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Fig. 3. Kinetics of dehydroquinase-modified by carbomethyl (CAK).

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Fig. 4. Stoichiometry of the inactivation of dehydroquinase by carbomethyl. Reaction mixture was incubated at a function of the number of carbomethyl groups incorporated per protein monomer. Incubation conditions were as described in Fig.2 A. Further details are presented in Experimental Procedures. Two experiments are shown in this figure (Fig. 3a). The enzyme (240 mg/ml) was incubated with carbomethyl [14C]arabinose and the residual enzyme activity was measured. Cleavage incorporation was measured after either (a) the TCA precipitation of samples into filter with or (b) dialysis of samples against phosphate buffer.

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Fig. 5. Comparison of HPLC trace profiles of dehydroquinase modified with carbomethyl [14C]arabinose and as described in Experimental Procedures. The enzyme (240 mg/ml) was incubated with carbomethyl [14C]arabinose and the residual enzyme activity was measured. Cleavage incorporation was measured after either (a) the TCA precipitation of samples into filter with or (b) dialysis of samples against phosphate buffer.
**Chemical Modification of Dehydroquinate**

**Fig. 1.** The chromatography of dehydroquinate TP1.
TP1 was chromatographed on C18 reverse-phase columns equilibrated with sodium phosphate (100 mM, pH 7.4) and developed with an aqueous to gradient; peaks were eluted in 20 min.

**Table 1.** Automated Edman degradation of the labeled peptide TP1.

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<th>Cycle Number</th>
<th>Residue</th>
<th>Yield (pmole)</th>
<th>CPM</th>
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<tbody>
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<td>1</td>
<td>Leu</td>
<td>2000</td>
<td>72</td>
</tr>
<tr>
<td>2</td>
<td>Val</td>
<td>1275</td>
<td>50</td>
</tr>
<tr>
<td>3</td>
<td>Ser</td>
<td>1440</td>
<td>50</td>
</tr>
<tr>
<td>4</td>
<td>Leu</td>
<td>60</td>
<td>121</td>
</tr>
<tr>
<td>5</td>
<td>Ala</td>
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<td>6</td>
<td>Lys</td>
<td>655</td>
<td>92</td>
</tr>
</tbody>
</table>

* Yld: yield of TP1 amino acid

**Table 2.** Automated Edman degradation of the labeled peptide TP2/CT.

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<th>Yield (pmole)</th>
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<tr>
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<td>Lys</td>
<td>203</td>
<td>107</td>
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</table>

* Yld: yield of TP2 amino acid

**Fig. 2.** Locations of labeled peptides TP1 and TP2/CT in dehydroquinate sequence.

The positions of the labeled peptides in the E. coli dehydroquinate sequence are given together with the residues of those regions with the same homology. The Edman sequence: (a) from Charlas et al. (1984), (b) from Duran et al. (1986), (c) from Duran et al. (1988), and (d) from Duran et al. (1989). TP1, tyrosyl peptide 1 (TP1); TP2, tyrosyl peptidase product 2 (TP2/CT). The predicted sequence replacements are indicated for E. coli.
Active site labeling of the shikimate pathway enzyme, dehydroquinase. Evidence for a common substrate binding site within dehydroquinase and dehydroquinate synthase.

C Kleanthous, D G Campbell and J R Coggins


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