Chemical Modification of Chalcone Isomerase by Mercurials and Tetrathionate

EVIDENCE FOR A SINGLE CYSTEINE RESIDUE IN THE ACTIVE SITE*

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Chalcone isomerase from soybean is inactivated by stoichiometric amounts of p-mercuribenzoate or HgCl₂. Spectral titration of the enzyme with p-mercuribenzoate indicates that a single thiol group is modified. Treatment of modified enzyme with KCN or thiols results in a complete restoration of enzyme activity demonstrating that the inactivation is not due to irreversible protein denaturation. A product of the enzymatic reaction, naringenin, provides complete kinetic protection against inactivation by both mercurials. The binding constant (33 μM) for naringenin determined from the concentration dependence of the protection agrees with the inhibition constant (34 μM) for naringenin as a competitive inhibitor of the catalytic reaction. This agreement demonstrates that the observed kinetic protection results from the specific binding of naringenin to the active site. Incubation of native chalcone isomerase with sodium tetrathionate (0.1 M) results in a slow time-dependent loss of enzymatic activity. The inactivation of chalcone isomerase by tetrathionate and N-ethylmaleimide becomes very rapid in the presence of 6 M urea, indicating that the native tertiary structure is responsible for the low reactivity of the enzymatic thiol. The stoichiometric modification of reduced and denatured chalcone isomerase by [3H]N-ethylmaleimide indicates that the enzyme contains only a single cysteine residue and does not contain any disulfides. The evidence presented suggests that the only half-cystine residue in chalcone isomerase is located in the active site and thereby provides the first clue to the location of the active site in chalcone isomerase.

Chalcone isomerase increases the rate constant for cyclization of chalcone (I) by 36 million-fold over that of the spontaneous reaction (5). However, little is known about the residues present in the enzymatic active site or about the mechanism(s) responsible for this rate enhancement. Chalcone isomerase has been shown to lose activity upon treatment with diethylpyrocarbonate (6) or iodacetamide (7). Although, the enzymes from C. max (8) and P. vulgaris (9) are inhibited by mercurials, the absence of inhibition by sodium tetrathionate was taken as evidence that a sulfhydryl group is not essential for activity (1, 7, 9). The recent availability of homogeneous enzyme (5) has made it possible to probe the active site structure of this enzyme using chemical modification techniques.

In this paper, we demonstrate stoichiometric modification of a single active site thiol of chalcone isomerase by mercurials. Further, the previously reported failure of tetrathionate to inhibit the enzyme (7, 9) is shown to result from a slow rate of reaction with the active site thiol rather than the formation of a modified enzyme which is active. The assignment of the only half-cystine residue in the enzyme to the active site provides the first tag for the location of the active site in the primary sequence of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Naringenin, p-hydroxymercuronbenzoate, HgCl₂, NEM, and bovine serum albumin were purchased from Sigma, Oanifluor, Protosol, and N-[ethyl-2-3H]ethylmaleimide were obtained from Du Pont-New England Nuclear. Glutathione, DTG, sodium dodecyl sulfate, and buffers were products of Research Organics. 2',4',4'-Trihydroxychalcone was prepared as described previously (5, 10). Milli-Q water was used in the preparation of all aqueous solutions.

Chalcone isomerase was isolated from the Williams-82 cultivar of soybeans (G. max) and purified to homogeneity (5). The protein concentration of pure enzyme was determined by the absorbance at 280 nm using an extinction coefficient of 0.73 (cm mg/ml)⁻¹ and a molecular weight of 24,000 (5). The standard assay for chalcone...

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isomerase activity was performed at 25 °C by monitoring the loss of the chalcone absorbance at 390 nm using 40 μM 2',4',4-trihydroxy-chalcone in 50 mM Tris chloride buffer (pH 7.6) containing 1% ETOH. One international unit of enzyme activity is defined as the amount of enzyme which catalyzes the loss of 1 μmol of 2',4',4-trihydroxy-chalcone in 1 min at 25 °C in 50 mM Tris chloride, pH 7.6 (5).

Stock solutions of flavonoids were prepared in 95% ETOH and their concentrations determined at pH 7.6 in 50 mM Tris chloride (containing 1% ETOH) using the following gravimetrically determined extinction coefficients: 2',4',4-trihydroxychalcone, ε380 = 29,400 M⁻¹ cm⁻¹, naringenin, ε280 = 24,000 M⁻¹ cm⁻¹. The concentration of pMB solutions were determined from the absorbance at 292 nm in 50 mM potassium phosphate buffer at pH 6.8 using ε = 16,900 (11).

Kinetics of Inactivation—Chalcone isomerase (20–300 nM) was incubated with pMB (200–2000 nM), HgCl₂ (100 nM), or sodium tetrathionate (1 mM, 100 mM) at 25 °C and at various pH values. Aliquots (2–100 μl) were withdrawn over time and assayed for chalcone isomerase activity. Control incubations lacking only the inactivating reagent were run in parallel. The activity of the experimental incubation solution (E) was divided by the activity of the control incubation (E₀) to correct for any small activity losses not due to the inactivating reagent. When the concentration of the inactivator was in a large excess over the enzyme, the pseudo-first order rate constant for inactivation was obtained by a linear least squares fit of the plot of In(E/E₀) versus time. When the concentration of inactivator was not in great excess over the enzyme concentration, a second-order rate constant (k_inact) for inactivation of the enzyme was obtained from a nonlinear least squares fit of the data to Equation 3 (13). The parameters a and b are the initial concentrations of the enzyme and inactivator, respectively.

\[
\frac{1}{(a-b)} \ln \left( \frac{b}{(b-a + a (E/E₀))} \right) = k_{\text{inact}} \cdot t
\]

The dissociation constant for ligands from protection experiments, Kᵦ, were obtained by a nonlinear least square fit of the observed second-order rate constant for inactivation, kᵦ, at various concentrations of the protectant, P, according to Equation 3. The fitted values Kᵦ and Kᵦₑ are the dissociation constant for enzyme-protectant complex and the rate constant for inactivation without protectant, respectively.

\[
k_{\text{inact}} = \frac{k_{\text{inact}}}{1 + \frac{[P]}{K_p}}
\]

**RESULTS**

**Inactivation by Mercurials—** Incubation of chalcone isomerase with mercurials pMB or HgCl₂ results in a time-dependent loss of the enzyme activity (Fig. 1). In order to obtain rates slow enough to measure conveniently, low concentrations of enzyme and concentrations of the mercurial in only a 2–3-fold excess of the enzyme were required. The inactivation of chalcone isomerase followed second-order kinetics, with the rate of inactivation proportional to both the concentration of enzyme and the concentration of the mercurial (data not shown). The second-order rate constants for inactivation, kᵦ, are given in Table I.

**Treatment of chalcone isomerase with a stoichiometric amount of either of the mercuric reagents results in a partial loss of enzyme activity.** Fig. 2 shows a direct relationship between the fraction of activity lost and the fraction of a molar equivalent of mercuric added. The addition of one or more molar equivalents of pMB or HgCl₂ results in a loss of

![Graph showing time-dependent inactivation of chalcone isomerase (CHI) by mercurials.](image)

**FIG. 1. Time-dependent inactivation of chalcone isomerase (CHI) by mercurials.** Chalcone isomerase (145 or 20 nM) was incubated with pMB (460 nM) or HgCl₂ (40 nM) at 25 °C and pH 6.85 in 50 mM MOPS buffer (I = 0.1 M (KCI)). The solid symbols show the relative enzyme activities (E/E₀) determined by the standard assay without KCN as described under “Experimental Procedures.” The open symbols show the enzyme activities obtained following a 1-min pretreatment of the enzyme with 0.5 mM KCN prior to the addition of the substrate. The lines through the solid points were obtained by a nonlinear least squares fit of the data to Equation 2 and yield second-order rate constants of 134 ± 0.03 and 9.3 ± 0.9 μM·min⁻¹ for pMB and HgCl₂, respectively.

**TABLE I**

| Additions to incubation mixture | pH | kᵦ | Kᵦ (naringenin) |
|--------------------------------|----|----|-----------------|
| pMB                           | 6.8 | 6.8 × 10⁻⁶ | 33 ± 6          |
| HgCl₂                          | 6.8 | 6.8 × 10⁻⁶ | 33 ± 3          |
| 2',4',4-Trihydroxychalcone      | 6.8 | 6.8 × 10⁻⁶ | 34 ± 6          |
| Na₂S₂O₅                        | 5.2a| <0.005 | 9.4a × 10⁻³     |
| Na₂S₂O₅ + 6 M urea             | 7.5 | 7.5 × 10⁻³ | >690        |
|                                | 9.6 | 9.6 × 10⁻³ | >690        |

a 50 mM MOPS buffer I = 0.1 M (KCI).

b 50 mM MES buffer I = 0.1 M (KCI).

c 50 mM HEPES buffer I = 0.1 M (KCI).

d 50 mM CHES buffer I = 0.1 M (KCI).
Genin, a product of the enzyme catalyzed cyclization, to an incubation containing either pMB or HgCl₂ results in a decrease in the rate of inactivation. A high concentration of naringenin (1.5 mM) reduces the rate constant for inactivation by over 35-fold. A dissociation constant for naringenin at the binding site from which it protects against both mercurials was obtained by varying the concentration of naringenin (0–250 μM) and fitting the observed second-order rate constants for inactivation, kobs, to Equation 3. Table I summarizes these dissociation constants along with the inhibition constant for naringenin determined from its competitive inhibition of the catalytic reaction under the same experimental conditions.

Treatment of Chalcone Isomerase with Sodium Tetrathionate (Na₂S₂O₄) — Incubation of chalcone isomerase with 0.1 M sodium tetrathionate shows no inactivation at pH 5.2 and very slow time-dependent inactivation (t₁/₂ = 13 h) at pH 7.5. Over 10-fold faster inactivation is seen when the incubation is performed at pH 9.4, consistent with the modification of a cysteine residue. The second-order rate constants for tetrathionate mediated inactivation of chalcone isomerase are given in Table I.

To test the hypothesis that tetrathionate rapidly modifies the cysteine residue of chalcone isomerase without complete loss of enzyme activity (see "Discussion"), the ability of other thiol reagents to completely inactivate the tetrathionate-treated enzyme was explored. Enzyme incubated with sodium tetrathionate (0.1 M at pH 5.2, 7.5, and 9.4) was periodically assayed for enzyme activity in the presence or absence of 0.8 mM pMB. A slow exponential decrease in enzyme activity was observed when the assay was performed in the absence of pMB (Table I). In the presence of pMB, the tetrathionate-treated enzyme was always completely inactivated. HgCl₂ and NEM were also capable of completely inactivating the tetrathionate-treated enzyme (data not shown).

Effect of Urea on Enzyme Activity — Chalcone isomerase is not catalytically active when assayed with 2',4',4'-trihydroxychalcone (40 μM) in 6 M urea (pH 7.6, 50 mM Tris). However, when an aliquot of the enzyme is removed and diluted 100-fold into the standard assay solution, rapid turnover of the substrate into product occurs. Approximately 50% of the original specific activity of the enzyme is rapidly recovered. Extended incubation of chalcone isomerase (4.7 μM) at 25 °C in 6 M urea, slowly leads to an irreversible loss of the ability to recover enzyme activity. The half-times for this irreversible loss of enzyme activity at pH 7.5 and 9.6 are 290 and 80 min, respectively. The rapid reactivation of urea-denatured enzyme on dilution into the standard assay provides a convenient method of monitoring the modification of the unfolded enzyme. Addition of sodium tetrathionate (1 mM, pH 7.5 or 9.6) to chalcone isomerase in 6 M urea, followed by a determination of activity on dilution into the standard assay, indicates that tetrathionate rapidly modifies (t₁/₂ < 1 min) the enzyme leading to a loss of greater than 99% of the enzyme activity.

Covalent Incorporation of [H] from [H]NEM into Chalcone Isomerase — Incubation of chalcone isomerase (4.7 μM) with [14C]NEM (1.4 mM) in the presence of 6 M urea resulted in a rapid loss of all enzyme activity in less than 1 min at pH 7.5 and 9.6. The covalent incorporation of radioactivity into denatured chalcone isomerase was studied under a variety of conditions (see Table II). At neutral pH, incorporation of radioactivity occurs within a minute without further incorporation over an hour period. However, at pH 9.6 further incorporation occurs after an initial burst. While NEM is selective for thiol groups, it can form stable adducts with amines (18, 19). At pH 7 the half-time for reaction of the thiol group of cysteine with NEM (1.4 mM) is only 0.5 s (20), while...
TABLE II
Incorporation of radioactivity from [3H]NEM into chalcone isomerase

| Additions to reaction mixture | pH | Time | [3H] | n°  |
|------------------------------|----|------|------|-----|
| Urea (6 M)                   | 7.2| 1-50 | 0.6 ± 0.2 | 2   |
| Urea (6 M) + DTT (0.4 mM)    | 7.2| 25-30| 1.1 ± 0.1 | 4   |
| Urea (6 M)                   | 9.6| 1    | 0.8 ± 0.3 | 4   |
| Guanidine thiocyanate (5.1 M) | 7.5| 10   | 0.7 ± 0.1 | 3   |
| Guanidine thiocyanate (5.1 M) + DTT (0.77 mM) | 7.5| 10   | 1.0 ± 0.5 | 2   |

* The number of determinations used in calculating the standard deviations.

† Enzyme was treated with DTT in guanidine thiocyanate for 1 h prior to addition of 3.5 mM of [3H]NEM.

The half-time for the reaction with the amino group of glycine is 9.3 days (21). As the pH is increased both reactions increase in rate. At pH of 9.6 the half-time for reaction with the amino group of glycine can be calculated to be about 66 min. The initial very rapid incorporation seen on treatment of denatured enzyme with [3H]NEM is attributable to modification of a cysteine residue, while it is likely that the additional incorporation seen on long incubations at pH 9.6 is a consequence of nonspecific modification of protein amino groups.

The slightly less than stoichiometric incorporation of radiolabel from [3H]NEM into chalcone isomerase denatured in urea or guanidine thiocyanate (Table II) may be due to a partial oxidation of the enzymatic sulfhydryl to a sulfenic acid (—SOH) or an intermolecular disulfide (19). Pretreatment of the enzyme with DTT in urea or guanidine thiocyanate prior to the addition of [3H]NEM results in the incorporation of 1 mol eq of radioactivity (Table II).

**DISCUSSION**

Enzymatic reactions involving the addition of a nucleophile to a double bond conjugated to a carbonyl group are an important class of biochemical reactions (23). Chalcone isomerase is unique in this class because it catalyzes an intramolecular reaction in which the same substrate molecule contains both the nucleophile as well as the double bond (see Equation 1). Several mechanisms have been proposed for the catalysis of this apparently simple chemical reaction. Hahlbrock and co-workers (24) propose a mechanism involving general acid base catalysis through an intermediate flav-3-en-4-ol. In contrast, Boland and Wong (6) suggest nucleophlic catalysis by an active site imidazole, followed by an SN2 displacement by the 2'-phenolate of the substrate. A full description of enzymatic catalysis must await an inventory of active site residues that may interact with substrates, intermediates, and products. Chemical modification provides a viable method of identifying amino acid residues that may play an essential role in the catalytic reaction. Enzymatic sulfhydryl groups were the initial target of our investigation, since they are the most reactive nucleophilic amino acid side chain, and there is precedent for their involvement in acid-base catalysis and covalent catalysis in proline racemase (25) and thymidylate synthase (26), respectively.

Cysteine forms very tight complexes with both organic and inorganic mercurials (16,27). Although mercurials are capable of interaction with protein ligands other than cysteine (28-30), they are highly selective for the thiol side chain of cysteine when incubated at low concentration and in the presence of salt (16,19,31). The ability to titrate the activity of chalcone isomerase with substoichiometric amounts of pMB or HgCl₂ and an essentially complete loss of enzyme activity on the addition of stoichiometric amount of these mercurials (Fig. 2) suggests that modification of a single sulfhydryl group is responsible for the loss of enzyme activity. The increase in absorbance at 250 nm on titration of the enzyme with pMB (Fig. 2B) allows a direct spectral monitoring of the complex formation. The leveling off of the absorbance increase after 1 eq of pMB indicates that only a single site of the enzyme is modified. The extinction coefficient for the complex, 8 × 10⁴ M⁻¹ cm⁻¹, is in agreement with a value of 7.5 × 10⁴ M⁻¹ cm⁻¹ reported for the glutathione mercaptide (32), offering support that the increase in absorbance at 250 nm is indeed due to the formation of an enzymatic mercaptide. These results provide direct evidence that pMB interacts with a single sulfhydryl group of the enzyme.

The interaction of mercury with chalcone isomerase is very tight since there is complete inactivation when 10 nM HgCl₂ is added to 7 nM enzyme. Therefore, the observed dissociation constant, [Hg][E]/[E-Hg], must be less than 10⁻³⁵ M. If the enzymatic thiolate displaces a chloride ligand of HgCl₂ then the calculated dissociation constant for the ES⁻¹HgCl complex would be less than 10⁻²⁴ M. This very tight interaction argues strongly that the inactivation by HgCl₂ results from an interaction with a sulfhydryl group.

The inactivation by pMB is not simply due to the steric bulk of the benzene moiety, since the smaller HgCl₂ also inactivates the enzyme. Addition of KCN to enzyme that was previously inactivated by either pMB or HgCl₂ results in a rapid and complete restoration of the catalytic activity (Fig. 1). Cyanide forms very tight complexes with inorganic and organic mercurials. The dissociation constants for Hg(CN)₂ and MeHgCN are 10⁻4.7 M⁻¹ and 10⁻18 M which are almost as tight as the analogous complexes with a cysteine thiolate which have dissociation constants of 10⁻4.5⁻⁵ M⁻¹ and 10⁻15.7 M⁻¹, respectively (16). Presumably, cyanide binds to the mercury atom in the thiolate complex resulting in a release of the sulfhydryl ligand. This ligand exchange mechanism allows the kinetics of the reaction to be facile, and the high concentration of cyanide relative to the enzyme thiolate shifts the equilibriuim to favor the free enzyme sulfhydryl and the cyanide-chelated mercurial. The regeneration of full enzyme activity indicates that the activity loss on mercaptide formation is not due to an irreversible denaturation of the modified enzyme.

Naringenin, a product of the enzymatic reaction, provides kinetic protection against the loss of enzyme activity by pMB and HgCl₂. The dissociation constant determined for naringenin at the site from which it protects agrees with the value of Kᵣ for naringenin as a competitive inhibitor of the catalytic reaction (Table I). This agreement indicates that the protection results from the binding of naringenin at the active site of the enzyme. The complete kinetic protection afforded by binding of naringenin suggests that is provides a direct sterically

2 Calculated from the data of Leslie (21) using a pK of 9.6 for the amino group of glycine (22).
block between the inactivating reagents and the enzymatic sulffhydryl group. These results provide evidence that both mercurials modify the active site cysteine residue of chalcone isomerase.

Previous workers have concluded that chalcone isomerase does not contain an essential cysteine residue based on the absence of enzyme inhibition in the presence of up to 0.1 M sodium tetrathionate (1, 7, 9). The implicit assumption involved in drawing this conclusion was that tetrathionate should very rapidly modify cysteine and lead to loss in activity if a thiol group was essential for catalytic activity. In order to determine if tetrathionate might modify the enzymatic cysteine residue without causing complete loss in enzyme activity, we looked for the ability of tetrathionate to protect against inactivation by other thiol reagents which completely inactivate the enzyme. Chalcone isomerase with its cysteine residue modified by tetrathionate should no longer be modifiable by other thiol reagents. If this tetrathionate-modified enzyme retained some catalytic activity, then treatment with another thiol reagent should no longer be able to completely inactivate the enzyme. Enzyme treated with tetrathionate can still be completely inactivated by pMB, demonstrating that tetrathionate does not produce a thiol modified enzyme which retains catalytic activity. The enzyme modified by tetrathionate retains no (<1%) catalytic activity.

Tetrathionate is a slow time-dependent inactivator of the chalcone isomerase. The rate constant for inactivation increases at high pH values as would be expected for reaction with an enzyme thiolate (Table I). The low reactivity of this active site thiol is also exhibited in its reaction with mercurials. Although pMB and HgCl₂ are very potent inactivators of chalcone isomerase with large second-order rate constants for inactivation (Table I), at pH 6.8 pMB inactivates the enzyme 330-fold slower than the reaction of pMB with β-mercaptopethanol (32). Incubation of chalcone isomerase in 0.8 M urea, increases the rate constant for tetrathionate-mediated inactivation of the enzyme by over 77,000-fold (Table I). The rapid inactivation of the urea-denatured enzyme indicates that the three-dimensional structure of the enzyme is responsible for the low reactivity of the cysteine residue in chalcone isomerase.

Several lines of evidence lead us to conclude that chalcone isomerase from G. max contains no disulfides and only a single cysteine residue. Titration of denatured enzyme with [³H]NEM results in a stoichiometric incorporation of radioactivity. The absence of additional incorporation when the enzyme is reduced and denatured strongly argues that the enzyme does not contain any disulfide bonds (Table II). Additional evidence for only a single cysteine in chalcone isomerase comes from titration with 5,5'-dithiobis(nitrobenzoic acid) in 6 M guanidine hydrochloride (5). The presence of only a single half-cystine was confirmed by HPLC quantitation of cysteic acid following performic acid oxidation and acid hydrolysis of chalcone isomerase (5).

A survey of 207 protein sequences showed a 2.8% frequency for the amino acid cysteine (33), which is over 6-fold greater than that present in chalcone isomerase. Very few other enzymes contain only a single half-cysteine residue in their sequence, human carbonic anhydrase (34), streptococcal proteinase (31), and yeast maltase dehydrogenase (35) being the only examples of which we are aware. The recently reported cDNA sequence of chalcone isomerase from P. hybrida (3) also shows an unusually low cysteine content (2 out of 241 residues or 0.8%). The low sulphydryl content of the enzyme may not be surprising from an evolutionary point of view considering that the chalcone substrate is a powerful electrophile and is known to react in solution with free thiols (36).

Studies presented in this paper demonstrate that soybean chalcone isomerase contains only one essential residue that contains a single cysteine residue and no disulfides. The modification of this residue by thiol reagents leads to complete loss of enzymatic activity, and the native tertiary structure of the enzyme dampens its reactivity relative to free cysteine. The assignment of the only half-cysteine residue to the active site provides the first clue to its location in the linear sequence of the enzyme and makes it tempting to postulate that this residue has a role in either the catalysis or regulation of this enzyme. Current studies are directed toward elucidating the function of the active site cysteine residue in chalcone isomerase.

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