Reactivity and pH Dependence of Thiol Conjugation to *N*-Ethylmaleimide: Detection of a Conformational Change in Chalcone Isomerase†

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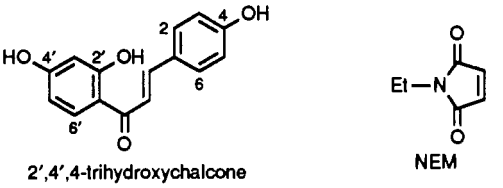
ABSTRACT: The reactivity of simple alkyl thiolates with *N*-ethylmaleimide (NEM) follows the Brønsted equation, $\log k_{S^-} = \log G + \beta pK$, with $G = 790\ M^{-1}\ min^{-1}$ and $\beta = 0.43$. The rate constant for the reaction of the thiolate of 2-mercaptoethanol with NEM is $10^7\ M^{-1}\ min^{-1}$, whereas the rate constant for the reaction of the protonated thiol is less than $0.0002\ M^{-1}\ min^{-1}$. The intrinsic reactivity of the protonated thiol (SH) is over (5×10^{10})-fold less than the thiolate (S^-) and makes a negligible contribution to the reactivity of thiols toward NEM. The rate of NEM modification of chalcone isomerase was conveniently measured by following the concomitant loss in enzymatic activity. The pseudo-first-order rate constants for inactivation show a linear dependence on the concentration of NEM up to 200 mM and yield no evidence for noncovalent binding of NEM to the enzyme. Evidence is presented demonstrating that the modification of chalcone isomerase by NEM is limited to a single cysteine residue over a wide range of pH. Kinetic protection against inactivation and modification by NEM is provided by competitive inhibitors and supports the assignment of this cysteine residue to be at or near the active site of chalcone isomerase. The pH dependence of inactivation of the enzyme by NEM indicates a pK of 9.2 for the cysteine residue in chalcone isomerase. At high pH, the enzymatic thiolate is only (3×10^{-5})-fold as reactive as a low molecular weight alkyl thiolate of the same pK, suggesting a large steric inhibition of reaction on the enzyme. As the pH of the medium is reduced, the reactivity of the enzymatic thiolate approaches that of a free alkyl thiolate, suggesting a conformational change that removes the steric inhibition. Alterations in the reactivity of sterically hindered protein thiolates provide a means of detecting conformational changes in proteins.

Understanding the effect of protein structure on the nucleophilic reactivity of amino acid side chains is a longstanding question in biochemistry. The thiol group of the amino acid cysteine is a very reactive nucleophile under physiological conditions and perhaps provides the best opportunity to understand how protein structure can modulate nucleophilic reactivity (Roberts et al., 1986). Chalcone isomerase (EC 5.5.1.6) from soybeans (*Glycine max*) has been shown to be a rare example of an enzyme that contains no disulfides and only a single cysteine residue at or near the active site (Bednar et al., 1989).

Because of the importance of thiol groups in biochemistry, substantial effort has been expended in designing specific reagents for their characterization and quantitation (Torchinsky, 1981; Brocklehurst, 1979; Liu, 1977; Friedman, 1973; Jocelyn, 1972). NEM[1] is a selective sulfhydryl group modifying reagent which contains a Michael acceptor similar to that in the chalcone substrate (see Chart I). Chalcone isomerase catalyzes the addition of the 2′-hydroxyl group of 2′,4′,4-trihydroxychalcone to the double bond conjugated to the carbonyl group to produce 4′,7-dihydroxyflavanone.

This paper, which establishes the dependence of the rate constant for the reaction of NEM with simple alkyl thiols and the active site cysteine of chalcone isomerase on their degree of protonation and pK, provides a basis for interpreting the reactivity of NEM with thiol groups in proteins. A protonated thiol (SH) is shown to be over (5×10^{10})-fold less reactive than the corresponding thiolate (S^-), suggesting that only the thiolate form will contribute to the reaction of NEM with protein thiols. The structure of a protein can greatly affect the reactivity of its amino acid side chains. Conversely, the reactivity of the amino acid side chains provides a probe of the structure of the protein. Using chalcone isomerase, we show that a pH-dependent conformational change in the protein can alter the reactivity of a protein thiolate by almost 5 orders of magnitude. The method developed here is a very sensitive means of detecting conformational changes in proteins.

Chart I



Experimental Procedures

Materials. Apigenin was purchased from Sigma. *N*-Ethyl[2,3-^{14}C]maleimide (10 mCi/mmol) and *N*-[*ethyl*-2-^{3}H]ethylmaleimide (40 Ci/mmol) were obtained from Am-

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[1] Abbreviations: apigenin, trivial name for 4′,5,7-trihydroxyflavone; naringenin, trivial name for 4′,5,7-trihydroxyflavanone; pMB, *p*-mercuribenzoate; NEM, *N*-ethylmaleimide; 4′,4-DHC, 4′,4-dihydroxychalcone; CAPS, 3-(cyclohexylamino)propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPSO, 3-(*N*-morpholino)-2-hydroxypropanesulfonic acid; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TAPS, 3-[[tris(hydroxymethyl)methyl]amino]propanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

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ersham and New England Nuclear, respectively. 4′,4-Dihydroxychalcone was prepared by the Claisen–Schmidt condensation of 4-hydroxybenzaldehyde with 4′-hydroxyacetophenone in alcoholic KOH (Geissman & Clinton, 1946). All chalcones were recrystallized several times from EtOH/H_2O and purified on a polyamide column eluting with 95% EtOH. Synthetic compounds constitute over 99% of the absorbance at 254 and 313 nm on reverse-phase HPLC analysis. Elution from an Alltech Econosphere C-18 column was achieved with aqueous 0.1 M acetic acid and methanol, either isocratically (nominally 55% MeOH) or by gradient elution (30% to 100% MeOH over 20 min at 1 mL/min). Stock solutions of flavonoids were prepared in 95% EtOH and their concentrations determined at pH 7.6 in 50 mM Tris-HCl (containing 1% EtOH) by using the following gravimetrically determined extinction coefficients: 2′,4′,4-trihydroxychalcone, ϵ_{390} = 29 400 M^{-1} cm^{-1}; 4′,4-dihydroxychalcone, ϵ_{362} = 26 000 M^{-1} cm^{-1}; naringenin, ϵ_{324} = 24 000 M^{-1} cm^{-1}. Other materials and methods have been described previously (Bednar & Hadcock, 1988; Bednar et al., 1989).

Chalcone isomerase was isolated from Williams-82 cultivar of soybeans (*G. max*) and purified to homogeneity (Bednar & Hadcock, 1988). The protein concentration of pure enzyme was determined by the absorbance at 280 nm by using an extinction coefficient of 0.73 (cm mg/mL)$^{-1}$ and a molecular weight of 24 000 (Bednar & Hadcock, 1988). The standard assay for chalcone isomerase activity was performed by monitoring the loss of the chalcone absorbance at 390 nm at 25 °C with 40 μM 2′,4′,4-trihydroxychalcone in 50 mM Tris-HCl buffer (pH 7.6) containing 1% EtOH. One international unit of enzyme activity is defined as the amount of enzyme that catalyzes the loss of 1 μmol of 2′,4′,4-trihydroxychalcone in 1 min at 25 °C in 50 mM Tris-HCl, pH 7.6 (Bednar & Hadcock, 1988).

Kinetics of Reaction of NEM with Low Molecular Weight Thiols. Pseudo-first-order rate constants for reaction of low molecular weight thiols with NEM were measured at 25 °C, I = 1.0 M (KCl), in nitrogen-purged aqueous HCl solutions containing 0.4 mM EDTA. The concentration of thiol was in a 100–1000-fold molar excess of the NEM, and the reactions were monitored by the loss of the NEM absorbance [ϵ_{302} = 620 M^{-1} cm^{-1} (Gregory, 1955)]. Pseudo-first-order rate constants were obtained by a nonlinear least-squares fit of absorbance data collected over 3–10 half-times to a single exponential. Second-order rate constants, k_{obsd}, were calculated by dividing the observed pseudo-first-order rate constants by the concentration of thiol. The concentration of the thiol was determined by titration with DTNB (Riddles et al., 1983). Evaluation of the second-order rate constants at several thiol concentrations verified that they were independent of the thiol concentration.

Kinetics of Reaction of Chalcone Isomerase with NEM. The rate of modification of the single cysteine residue of chalcone isomerase by NEM was determined by monitoring the inactivation of the enzyme. Chalcone isomerase (20–300 nM) was incubated with NEM (0.1–200 mM) at a variety of 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 (E_c) to correct for any small activity losses not due to the inactivating reagent. At pH <7.5, the pseudo-first-order rate constant for inactivation was obtained by a linear least-squares fit of the plot of ln (E/E_c) vs time.

Since NEM is not stable in alkaline solutions, the rate constant for decomposition of NEM at pH values between 7.4 and 10.5 in 50 mM TAPS, CHES, or CAPS buffer was determined by monitoring the loss of the absorbance at 302 nm. Plots of ln $[[A(t) - A(\infty)]/[A(0) - A(\infty)]]$ vs time were linear for at least 3 half-lives. The slopes of these plots yield the first-order rate constants for decomposition (k_{decomp}) of NEM. The activity of hydroxide, a_{OH}, was determined from the pH meter reading and a pK_w of 13.8 (Serjeant, 1984).

The observed pseudo-first-order rate constant for inactivation of the enzyme (k_{obsd}) by NEM at pH >7.5 was obtained by fitting the fractional enzyme activities (E/E_c) as a function of time according to eq 1, which accounts for the decomposition of the NEM. The second-order rate constant for inactivation, k_{inact}, was obtained by dividing k_{obsd} by the [NEM].

$$\ln (E/E_c) = (-k_{obsd}/k_{decomp})(1 - e^{-k_{decomp}t}) \quad (1)$$

The dissociation constants for ligands from protection experiments, K_I, were obtained by a nonlinear least-squares fit of the observed first-order rate constant for inactivation, k_{obsd}, at various concentrations of the protectant, P, according to eq 2. The fitted values of K_I and k_{inact} are the dissociation constant for enzyme–protectant complex and the rate constant for inactivation without protectant, respectively.

$$k_{obsd} = k_{inact}[NEM]/(1 + [P]/K_I) \quad (2)$$

Results

Reaction of NEM with Low Molecular Weight Thiols. The pH dependence of the observed second-order rate constant (k_{thiol}) for the reaction of NEM with thiols containing no other ionizable groups should be given by eq 3, where K represents the acid dissociation constant for the thiol group and k_{S^-} and k_{SH} represent the rate constants for reaction of NEM with the thiolate anion and the protonated thiol group, respectively. Gorin et al. (1966) studied the reaction of NEM with cysteine in the pH range 3–5 and found a small poorly determined term which they attributed to reaction of the protonated thiol. In order to determine the value of k_{SH}, we measured the rate constants for the reaction of simple alkyl thiols with NEM at lower pH. When $[H^+] \gg K$, eq 3 reduces to eq 4.

$$k_{thiol} = \frac{Kk_{S^-}}{[H^+] + K} + \frac{[H^+]k_{SH}}{[H^+] + K} \quad (3)$$

$$\frac{[H^+]k_{thiol}}{K} = k_{S^-} + \frac{[H^+]k_{SH}}{K} \quad (4)$$

Values of $[H^+]k_{thiol}/K$ for the reaction of 2-mercaptoethanol are equal to (11 ± 1) × 10^6 M^{-1} min^{-1} and were found to be independent of pH in the pH region of 0–2. The constancy of this value indicates the protonated thiol, k_{SH}, makes no significant contribution to the observed reaction rate. Thus, for all practical purposes reaction of a thiol group with NEM can be assumed as occurring via the thiolate anion. Allowing for experimental error (±10%), an upper limit on the value of the rate constant for the protonated thiol, k_{SH}, of 2 × 10^{-4} M^{-1} min^{-1} can be calculated. The protonated thiol of 2-mercaptoethanol is, therefore, over 10 orders of magnitude less reactive toward NEM than the thiolate anion.

Table I shows the thiol pK dependence of the second-order rate constant (k_{S^-}) for the attack of thiolate anions on NEM. The experimentally determined dependencies of rate constant on thiolate basicity (thiol pK) are fit by the Brønsted equation:

$$\log k_{S^-} = \log G + \beta pK \quad (5)$$

Table I: Dependence of the Second-Order Rate Constant for Thiolate Anion Reaction with NEM on the pK of the Thiol

thiol	pK	k_S-[a] (M^{-1} min^{-1})
2-mercaptoethanol	9.61[b]	(11 ± 1) × 10^6
2-mercaptoethylamine[c]	8.46[d]	(4.1 ± 0.1) × 10^6
L-cysteine ethyl ester[c]	7.30[e]	(1.10 ± 0.05) × 10^6

[a] Rate constants determined from $[H^+]k/K$ over the pH range 0–2 at 25 °C, I = 1.0 M (KCl). The rate constants are independent of pH. [b] Jencks and Salvesen (1971). [c] pK and rate constant for the species with a protonated amino group. [d] Lewis et al. (1980). [e] Reuben and Bruice (1976).

with log G = 2.90 ± 0.06 and β = 0.43 ± 0.03 (plot not shown). The Brønsted equation (eq 5) taken together with eq 3 (where k_{SH} = 0) yields eq 6 (Roberts et al., 1986). This

$$\log k_{thiol} = \log G + \beta pK + \log K - \log ([H^+] + K) \quad (6)$$

equation predicts the second-order rate constant for an alkyl thiol with NEM at any pH.

Inactivation of Chalcone Isomerase by NEM. Incubation of chalcone isomerase with NEM at pH values less than 7.5 results in a pseudo-first-order time-dependent loss of enzyme activity (Figure 1A). At higher pH values the semilogarithmic plots are not linear. The curvature is attributable to a base-dependent decomposition of NEM (Gregory, 1955). The rate constant for decomposition of NEM (k_{decomp}) was determined directly by monitoring the loss of NEM absorbance in buffered solution. The rate constant for decomposition of NEM divided by the activity of hydroxide, k_{decomp}/a_{OH}, is 1740 ± 120 M^{-1} min^{-1} and 2540 ± 180 M^{-1} min^{-1} in the pH ranges 7.4–9.0 (50 mM TAPS buffer) and 9.3–10.5 (50 mM CHES and CAPS with I = 0.1 M), respectively. The curvature in plots of the time-dependent loss of enzyme activity resulting from NEM (see Figure 1A) is well fit by eq 1 which allows for the decomposition of the inactivating reagent.

NEM contains an α,β-unsaturated carbonyl moiety similar to that present in the chalcone substrate (Chart I). In order to test for possible noncovalent binding of NEM to the enzyme prior to inactivation, the dependence of the rate constant for inactivation on the concentration of NEM was explored. Figure 1B shows a linear increase in the observed first-order rate constant (k_{obsd}) for inactivation even up to 200 mM NEM. The absence of curvature indicates that the dissociation constant for any noncovalent NEM–enzyme complex that might form is much greater than 200 mM. The slope of the plot in Figure 1B yields the second-order rate constant for inactivation (k_{inact}). The dependence of k_{inact} on pH is shown in Figure 1C.

Kinetic Protection against Inactivation and Covalent Incorporation. Mercurials have been shown to selectively modify the single active site cysteine residue of chalcone isomerase (Bednar et al., 1989). Prior modification of the cysteine residue of chalcone isomerase by $HgCl_2$ or pMB results in a greater than 10-fold protection against inactivation by NEM (Table II). Chalcone isomerase modified with a stoichiometric amount of pMB or $HgCl_2$ shows no covalent incorporation of radioactivity when treated with [^{3}H]NEM (Table III). Table III also shows that 4′,4-dihydroxychalcone, a competitive inhibitor of the active site, also provides protection against inactivation and covalent incorporation which would normally result from incubation of the enzyme with [^{3}H]NEM.

Addition of naringenin, a product of the enzyme reaction, to an incubation mixture of the enzyme and NEM results in a decrease in the rate constant for inactivation. A dissociation constant for naringenin at the binding site from which it protects against inactivation by NEM was obtained by varying the concentration of naringenin (0–200 μM) and fitting the observed first-order rate constants for inactivation, k_{obsd}, to eq 2. Table II summarizes these dissociation constants along with the inhibition constant for naringenin determined from

Table II: Rate Constants for Inactivation of Chalcone Isomerase by Thiol Reagents, Protection against Inactivation, and Reactivity Relative to a Free Alkyl Thiol[a]

additions to incubation mixture	pH	k_{inact} (M^{-1} min^{-1})	K_I(naringenin)[b] (μM)	k_{inact}/k_{thiol}[c]
NEM	6.8[d]	8.5 ± 0.3	30 ± 3	
2′,4′,4-trihydroxychalcone	6.8[e]		34 ± 6[f]	
(E + 2 μM pMB) + NEM	6.8[g]	0.3 ± 0.8[h]		
(E + 2 μM $HgCl_2$) + NEM	6.8[g]	0.0 ± 0.6[h]		
NEM	8.5[i]	57 ± 2	54 ± 13	
2′,4′,4-trihydroxychalcone	8.5[i]		54 ± 8[f]	
naringenin (1.5 mM) + NEM	8.5[i]	2 ± 2		
apigenin[j] (39 μM) + NEM	8.5[i]	5 ± 1		
(E + 6 M urea) + NEM	5.8[k]	2800 ± 1000		0.9 ± 0.4
	6.8–9.5	>6000		
iodoacetamide[l]	5.2[k]	0.06		0.3
	9.3[m]	0.094		8.1 × 10^{-5}
pMB[n]	5.2[k]	2.5 × 10^6		8.3 × 10^{-3}
	9.4[m]	1.0 × 10^5		3.7 × 10^{-7}

[a] Chalcone isomerase was incubated with the indicated compounds at 25 °C, and the rate constant for inactivation, k_{inact}, was determined as described under Experimental Procedures. [b] The dissociation constant for naringenin at the site from which it protects, K_I, was determined by a nonlinear least-squares fit of the rate consatnt for inactivation, k_{obsd}, at various concentrations of the protectant, P, according to eq 2. [c] The value of k_{thiol} was calculated from eq 3 by using a pK of 9.16 and rate constants obtained from model thiol as indicated in the footnotes. [d] 100 mM PIPES buffer I = 0.15 M (KCl). [e] 50 mM MOPSO buffer I = 0.1 M (KCl). [f] The inhibition constant for naringenin as a competitive inhibitor against the substrate 2′,4′,4-trihydroxychalcone was determined from a series of initial velocity data at six different substrate concentrations and five different fixed concentrations of naringenin. [g] 5 mM potassium phosphate containing 50 mM K_2SO_4. [h] Rate constant for inactivation by NEM. The mercurial inactivated enzyme was reactivated by a 1-min pretreatment with 0.5 M KCN prior to the standard activity assay (Bednar et al., 1989). [i] 50 mM TAPS buffer I = 0.1 M (KCl). [j] Apigenin is reported to be a competitive inhibitor of chalcone isomerase with an inhibition constant of 4.4 μM at pH 7.6 and 30 °C (Boland & Wong, 1975). [k] 50 mM MES buffer I = 0.1 M (KCl). [l] The rate constant for the reaction of the thiolate (k_s-) of glutathione with iodoacetamide is about 2000 M^{-1} min^{-1} (Guidotti, 1967; Halasz & Polgar, 1976; Dahl & Mckinley-McKee, 1981). [m] 50 mM CHES buffer I = 0.1 M (KCl). [n] The rate constants for the reaction of 2-mercaptoethanol with pMB are k_s- = 4.5 × 10^{11} M^{-1} min^{-1} and k_{SH} = 2.5 × 10^8 M^{-1} min^{-1} (Hasinoff et al., 1971).

Table III: Effect of Added Ligands on the Incorporation of Radioactivity from [^{3}H]NEM into Chalcone Isomerase[a]

additions to reaction mixture	pH	time (min)	mol of ^{3}H/mol of enzyme	N[b]
none	5.7	150, 250, 500	0.9 ± 0.1	3
pMB (5.1 μM)	5.7	150, 250, 500	0.00 ± 0.06[c]	3
$HgCl_2$ (5.1 μM)	5.7	150, 250, 500	0.00 ± 0.02[c]	3
4′,4-DHC (50 μM)	5.7	150, 250, 500	0.15 ± 0.03[d]	3
4′,4-DHC (2 mM)	9.3	20–40	0.09 ± 0.08[e]	7

[a] Chalcone isomerase (5 μM) was incubated with the indicated ligands and [^{3}H]NEM (1.4 mM) at the indicated pH. The incorporation of ^{3}H was determined after the indicated time by a filter disk method described previously (Bednar et al., 1989). [b] The number of determinations used in calculating the standard deviations. [c] No irreversible loss of enzyme activity resulting from NEM was detected. [d] The average residual activity is 95 ± 8%. [e] The average residual activity is 100 ± 10%.

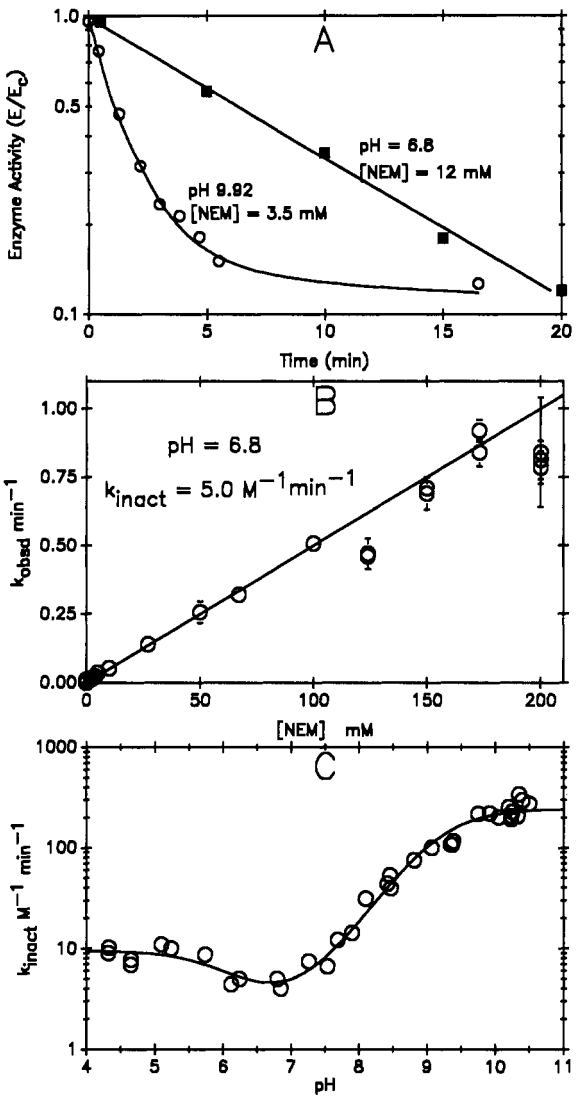


FIGURE 1: Inactivation of chalcone isomerase by NEM. (A) Chalcone isomerase (0.75 μM) was incubated with NEM at 25 °C in 50 mM buffer [I = 0.1 M (KCl)]. (■) pH 6.8 and [NEM] = 12 mM. (○) pH 9.92 and [NEM] = 3.5 mM. The activity of the enzyme divided by the activity of a control without NEM (E/E_c) was determined as described under Experimental Procedures. The staight line is a least-squares fit to the ln (E/E_c) vs time and yields an observed rate constant of 0.11 min^{-1}. The curved line is a nonlinear least-squares fit to eq 1 and yields a first-order rate constant for inactivation (k_{obsd} = 0.77 min^{-1}) and a rate constant for the first-order decomposition of NEM (k_{decomp} = 0.36 min^{-1}). (B) Chalcone isomerase was incubated with the indicated concentrations of NEM at 25 °C and pH 6.8 in 50 mM MOPSO buffer [I = 0.1 M (KCl)] and assayed for enzyme activity as a function of time according to the standard assay. The observed pseudo-first-order rate constant for inactivation, k_{obsd}, was obtained from the slope of a plot of the ln of enzyme activity vs time as in (A) above. The slope of the plot of k_{obsd} vs [NEM] yields the second-order rate constant for inactivation, k_{inact}. (C) Chalcone isomerase was incubated with NEM at the indicated pH values. The second-order rate constant for inactivation, k_{inact}, was obtained as indicated in (A) and (B) above. The solid line is obtained from a nonlinear least-squares fit of the data to eq 7, with the following fitted parameters: pK_A = 3.2 (not fit), pK_B = 5.9 ± 0.3, pK_C = 9.16 ± 0.07, k_b = 9.5 ± 1.0 M^{-1} min^{-1}, k_c = 2.9 ± 0.9 M^{-1} min^{-1}, and k_d = 245 ± 17 M^{-1} min^{-1}.

its competitive inhibition of the catalytic reaction under the same experimental conditions. The agreement of these dissociation constants demonstrates that the protection results from binding of naringenin to the active site. These data support the conclusion that the single cysteine residue of chalcone isomerase is located at or near the active site (Bednar et al., 1989).

Stoichiometry of the Covalent Modification of Chalcone Isomerase by NEM. The extent of covalent modification of chalcone isomerase by NEM was determined by incubation of native enzyme with radiolabeled NEM. Chalcone isomerase (5–15 μM) was incubated at 25 °C and pH 6.8 or 9.5 with radiolabeled NEM ([^{14}C]NEM (64 μM, 10 cpm/pmol) or [^{3}H]NEM (0.2–1.45 mM, 60 cpm/pmol)). At selected times aliquots (2–8 μg) were removed, the enzymatic activity determined by the standard assay, and the covalent incorporation determined by Cl_3CCOOH precipitation of the protein on a filter disk as described previously (Bednar et al., 1989). A one to one correlation between the loss in enzyme activity and the incorporation of covalently bound radioactivity into the enzyme was observed (data not shown). Complete inactivation of the enzyme is correlated with the stoichiometric incorporation of radioactivity at both low- and high-pH values.

When enzyme is completely inactivated by NEM under native conditions (pH 5.7 or 9.5) and then treated with [^{3}H]NEM under denatured conditions (8 M urea or 6 M guanidine thiocyanate), no further incorporation of radioactivity is detected. The absence of additional modification of the denatured enzyme suggests that the single cysteine residue of chalcone isomerase was already modified under native conditions.

Identification of the Product of the Reaction of NEM with Chalcone Isomerase. If NEM is reacting with the single cysteine residue of chalcone isomerase, then the expected product (after acid hydrolysis of the protein) is *S*-succinylcysteine (Lundblad & Noyes, 1984). Chalcone isomerase was inactivated by NEM under native conditions at low pH (5.7) and high pH (9.5). The amount of *S*-succinylcysteine was determined after hydrolysis and derivatization by analysis of PTH-amino acids on reverse-phase HPLC (Bednar & Haddcock, 1988). Authentic *S*-succinylcysteine standard was prepared by reaction of *N*-acetylcysteine with NEM, followed by acid hydrolysis and derivatization by the same procedure used for the protein. The average of four analyses at low and high pH yielded 100 ± 10% of the expected amount of *S*-succinylcysteine, indicating the NEM is indeed reacting specifically with the cysteine residue of chalcone isomerase.

Discussion

Cysteine residues play a number of important roles in biochemistry (Torchinsky, 1981; Liu, 1977; Friedman, 1973; Jocelyn, 1972), in part due to their thiol side chain of high intrinsic nucleophilicity. However, not all protein thiol groups exhibit a high reactivity, since the structure of protein can dramatically affect side-chain accessibility and protonation state. Consequently, the change in the reactivity of a protein thiol provides a means of detecting a change in protein structure. In this paper, we show that a large change in enzymatic thiolate reactivity can be used as a very sensitive probe for a conformational change in the protein structure as exemplified with the enzyme chalcone isomerase.

The high nucleophilic reactivity of thiols serves as the basis for the fairly common practice of selective chemical modification of cysteine residues. A number of chemical modification reagents are available that react selectively with thiols and make cysteine modification a particularly attractive target for monitoring conformational changes. NEM is a commonly used thiol-selective reagent which has been used to modify many proteins (Lundblad & Noyes, 1984; Webb, 1966b). Although some model studies have been reported (Gorin et al., 1966; Semenow-Garwood, 1972; Torchinsky, 1981), little is know about the intrinsic reactivity of thiolates toward NEM and the relative reactivity of the protonated thiol (SH) and

its corresponding thiolate (S^-). In order to probe the reactivity of a protein thiol with NEM, a more detailed understanding of the reactivity of NEM toward simple low molecular weight thiols was necessary.

Our studies of the kinetics of the reaction of NEM with low molecular weight alkyl thiols allow us to predict the intrinsic reactivity of a thiolate on the basis of the pK of the thiol (eqs 5 and 6). Further, we have demonstrated that the protonated thiol undergoes no detectable reaction with NEM, contrary to that reported previously (Gorin et al., 1966). The protonated thiol (SH) is at least (5×10^{10})-fold less reactive than the corresponding thiolate anion (S^-). The absence of any reactivity of the protonated thiol greatly simplifies the interpretation of the pH dependence of the modification of an enzymatic cysteine, since one can reasonably assume that all of the observed reaction will be due to thiolate forms of the enzyme (ES^-) and not to any of the protonated thiol forms of the enzyme (ESH).

Chalcone isomerase contains no disulfides and only a single cysteine residue located at or near the active site (Bednar et al., 1989). The simplicity of this enzyme makes it a good candidate for probing the relationship between protein structure and thiol group reactivity. As documented under Results, NEM inactivates chalcone isomerase (Figure 1, Table II) with stoichiometric modification of the enzymatic cysteine residue. Therefore, the modification of the enzymatic thiol is conveniently measured by the loss in enzyme activity.

The pH dependence of modification shows a plateau at high pH and decreases as the pH is lowered, suggesting a pK of 9.2 for the thiol group of a single cysteine residue of chalcone isomerase (Figure 1C). The thiol-selective reagent NEM bears some structural similarity to the chalcone substrate (Chart I) and binding of the reagent to the enzyme is a possibility. Such binding could tend to shift the pK observed in chemical modification studies from the true pK (Schmidt & Westheimer, 1971; Knowles, 1976). The absence of any significant noncovalent binding ($K_D > 200$ mM) of NEM to the enzyme (Figure 1B) suggests that a true macroscopic pK is observed. Comparing the value of this pK to that of glutathione (9.0) and 2-mercaptoethanol (9.6) suggests that it is not significantly perturbed by the structure of the protein (Rabenstein, 1973; Jencks & Salvesen, 1971).

The rate constant for reaction of the thiolate form of the enzyme (ES^-) at pH >8 is only (3×10^{-5})-fold of the value predicted from model studies for a simple alkyl thiolate of the same pK (Figure 2). A number of reasons have been given for the decreased reactivity of amino acid side chains on proteins such as ionization state, hydrogen bonding, electrostatic effects, hydrophobic interactions, and steric effects (Webb, 1966a; Torchinsky, 1981; Boyer, 1959; Friedman, 1973). Most of these interactions should yield only a modest effect on reactivity except for steric effects which potentially could results in a very large decrease in reactivity. At high pH, a 10^4- to 10^6-fold slower rate of inactivation than expected for reaction of a free alkyl thiolate is also observed with the thiol reagents iodoacetamide and pMB (Table II). This large and similar degree of inhibition seen with all thiol reagents studied is consistent with the reduced reactivity being a result of steric factors (Boyer, 1959). Further, denaturation of the enzyme in urea leads to a large increase in the thiolate reactivity predicted from elimination of steric effects (Table II). We conclude therefore that the structure of the protein at pH >8 provides a significant steric inhibition of the reaction of the enzymatic thiolate.

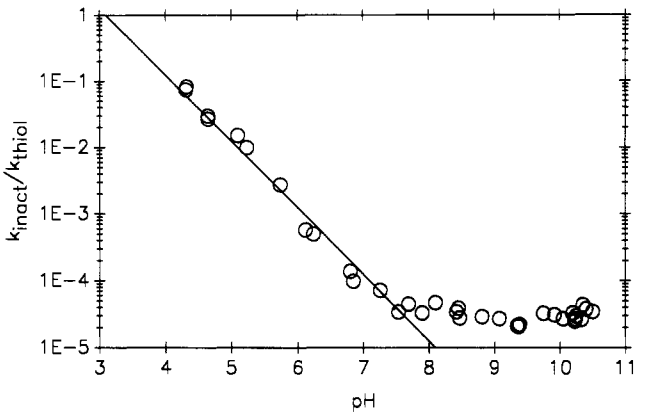


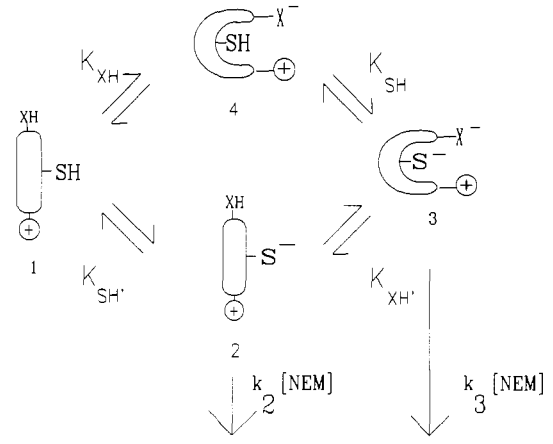
FIGURE 2: Dependence on pH of the reactivity of the active site cysteine of chalcone isomerase with NEM relative to a simple alkyl thiol. The rate constant for inactivation of chalcone isomerase by NEM, k_{inact}, was divided by the rate constant, k_{thiol}, for reaction of NEM with a simple alkyl thiol. The value of k_{thiol} was calculated from eq 6 by using a pK = 9.16, β = 0.43, and log G = 2.09. The line is drawn with a slope of 1.

As the pH is decreased below the pK of the enzymic thiol, the rate constant for inactivation was expected to decrease to zero. The absence of any further decrease in the rate constant for inactivation of chalcone isomerase at pH values less than 8 was initially very surprising (Figure 1C). The rate constant for this low pH plateau is only 20-fold less than for the high-pH plateau. It is unlikely that this represents a reaction of the protonated thiol since model studies indicate that this form is over 10 orders of magnitude less reactive that the thiolate form. This pronounced pH-independent region at low pH must represent an additional term in the rate law for inactivation of the enzyme.

Although NEM is a selective modifier of cysteine residues, potentially it could react with other amino acid residues (Smyth et al., 1964; Brewer & Riehm, 1967; Leslie, 1970) especially if the native structure of the protein accelerated the reaction. It was conceivable that modification of another type of amino acid residue might explain the inactivation of the enzyme at pH values less than 8. Therefore, it was necessary to rigorously demonstrate that NEM inactivates the enzyme by only modifying the single cysteine residue of chalcone isomerase under all our experimental conditions. Several lines of evidence, detailed under Results, support this conclusion. (1) The inactivation of chalcone isomerase is directly correlated to the covalent incorporation of 1 molar equiv of radioactivity from NEM at both low and high pH. (2) Addition of 1 equiv of pMB or $HgCl_2$/mol of chalcone isomerase, which selectively modifies the active site cysteine of chalcone isomerase (Bednar et al., 1989), affords substantial protection against the inactivation (Table II) and the covalent modification (Table III) caused by NEM. (3) Chalcone isomerase inactivated by NEM under native conditions shows no further incorporation of radioactivity when treated with [^{3}H]NEM under denatured conditions, suggesting that the single cysteine residue of chalcone isomerase was already modified under native conditions. (4) Finally, acid hydrolysis of enzyme inactivated under native conditions at pH 5.7 and 9.5 yields 1 molar equiv of *S*-succinylcysteine, demonstrating that NEM is reacting with a cysteine residue over a wide range of pH. These results demonstrate that the independence of k_{inact} at low pH (Figure 1C) indeed results from the modification of the enzymatic cysteine.

Insight into the pH dependence of k_{inact} can be obtained by considering the model studies. In Figure 2 the rate constant for inactivation of the enzyme by NEM is divided by the

Scheme I



expected rate constant for reaction of a simple alkyl thiol with the same pK as the enzymatic thiol. As the pH is lowered below 8, there is an enormous increase in the relative reactivity of the *enzymatic thiolate*. The rate constant calculated for the enzymatic thiolate at low pH approaches that of a low molecular weight thiolate. It is as if at high pH the enzymatic thiolate exists in a "closed form" that has a very low reactivity, but at low pH the enzymatic thiolate exists in an "open form" that has a reactivity equivalent to that of a simple alkyl thiolate.

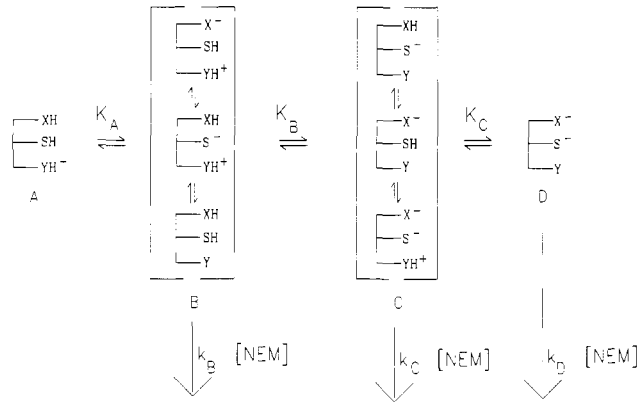
Scheme I shows the minimal scheme that can explain these data. Our model studies indicate that only thiolate forms of the enzyme are capable of reacting with NEM and it is therefore necessary that there be at least two thiolate forms of the enzyme. The simplest physical interpretation of this model is that there is a conformational change associated with the protonation of an X group that removes the steric inaccessibility of the enzymatic thiolate. Because of the large steric inhibition observed in the "closed form", this method is very sensitive and can detect small concentrations of the "open" enzyme conformation. For example, at pH 5 1 molecule in 100 exists in the "open" conformation and at pH 7 only 1 molecule in 10 000 of the enzyme exists in the "open" conformation.

Whereas Scheme I nicely explains the pH independence of k_{inact} at low and high pH values (Figure 1C), it does not explain the dip in k_{inact} seen around pH 7. Although this is only a small drop, it appears to be outside of experimental error. This small perturbation can be explained by postulating that the protonation of two groups is coupled to the conformational change, which increases the reactivity of the thiolate at low pH (Scheme II). The solid line in Figure 1C is a nonlinear least-squares fit to eq 7, which mathematically describes the model presented in Scheme II.

$$k_{inact} = \frac{k_B[H^+]/K_B + k_C + k_D K_C/[H^+]}{[H^+]^2/K_A K_B + [H^+]/K_B + 1 + K_C/[H^+]} \quad (7)$$

Formally, these models do not require any change in conformation of the protein other than addition and removal of protons. The shifts in the observed reactivity would then result from a change in the pK of the thiol caused by the protonation state of the X group and a change in the intrinsic reactivity of the thiol due to a change in its pK. There is precedent for this model in the case of papain, in which the active site cysteine residue directly interacts with a histidine residue (Roberts et al., 1986). If we assume that a direct interaction also occurs in chalcone isomerase, the intrinsic pK's of the SH group would need to be 6.2 and 3.2 when the X group was

Scheme II



unprotonated and protonated, respectively.[2] These are unusually low pK's for a thiol group in a protein (Tanford, 1962) which are normally in the range of 8–11. Low-pK thiol groups have been observed in papain (Roberts et al., 1986), bovine serum albumin (Lewis et al., 1980), and phosphoenolpyruvate carboxy kinase (Lewis et al., 1989). However, the reactivity of each of these thiols appears to be at least as great as expected for a simple alkyl thiolate. In light of the large steric inhibition for the reaction of the cysteine in chalcone isomerase, it seems more reasonable to postulate a normal-pK cysteine and a large increase in reactivity due to loss of the steric inhibition.

Several lines of evidence support assignment of the observed reactivity changes to a loss of steric inhibition at low pH. (1) A conformational change can increase the reactivity of the enzymatic thiolate, since denaturation of the enzyme in urea increases the reactivity of the enzyme thiolate to that expected for a free alkyl thiolate (Table II). (2) The pH dependence of the inactivation of chalcone isomerase by other thiol-selective chemical modification reagents reveals the same pattern seen with NEM in Figure 2. At high pH, the thiolate of chalcone isomerase is only 10^4- to 10^6-fold as reactive as a free alkyl thiolate toward the reagents pMB and iodoacetamide (Table II). However, at pH 5.2, the thiolate of the enzyme is no more than 10^2-fold less reactive than the model systems. The agreement among chemical modification reagents strongly supports the assignment of the altered reactivities being due to steric effects (Boyer, 1959). (3) A very short incubation of enzyme at pH 3 followed by a jump to pH 7.6 on dilution into the activity assay leads to a lag in the turnover of the enzyme before full enzyme activity is obtained. This is consistent with an altered conformation at pH 3 which takes 10's of seconds to return to the pH 7.6 conformation. It is possible that the conformational change observed here is a reversible

[2] The calculation was based on Scheme I with $k_3 = 245\ M^{-1}\ min^{-1}$ and macroscopic pK's of $pK_1 = 3.2$ and $pK_2 = 9.2$. The value for k_2 was calculated by assuming $\beta = 0.43$ describes the change in intrinsic reactivity of the enzymatic thiolate with pK. The best fit of the dependence of k_{inact} as a function of pH was obtained by adjusting the value for $K_{SH'}$ in the equation:

$$k_{inact} = \frac{K_{SH'}k_2/K_1 + K_2k_3/[H^+]}{1 + [H^+]/K_1 + K_2/[H^+]}$$

The microscopic pK's and the value of k_2 were obtained from the relationships:

$$K_1 = K_{XH} + K_{SH'}$$

$$K_2^{-1} = K_{SH}^{-1} + K_{XH'}^{-1}$$

$$k_2 = k_3[10^{\beta(pK_{SH'} - pK_{SH})}]$$

unfolding of the enzyme. At very low pH the enzyme would be expected to exist in the unfolded form and have the same reactivity as a simple alkyl thiolate. Only 0.01% of the enzyme is in the unfolded form at pH 7, and it is this form of the enzyme that reacts with NEM to produce the observed rate of inactivation.

The studies presented in this paper provide a basis for understanding the pH dependence of the reactivity of NEM with protein thiolates. We demonstrate that only the thiolate form (S^-) and not the protonated thiol form (SH) of a sulfhydryl group will react with NEM. Using the enzyme chalcone isomerase, we have shown that a large change in thiolate reactivity can be used as a sensitive probe for conformation changes in proteins. This method will be useful for proteins that have a sterically hindered thiolate whose "exposure" is altered by a change in conformation, whether caused by binding of an added ligand or by a change in pH. Further, the selective chemical modification of any group that is sterically very hindered in the native protein structure would provide a very sensitive probe for detecting unfolded protein and for studying the folding of proteins. While determination of the exact role of the cysteine residue and of the described conformational change of chalcone isomerase awaits further studies, the method used here should prove useful in the detection of conformational changes and the folding of proteins.

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