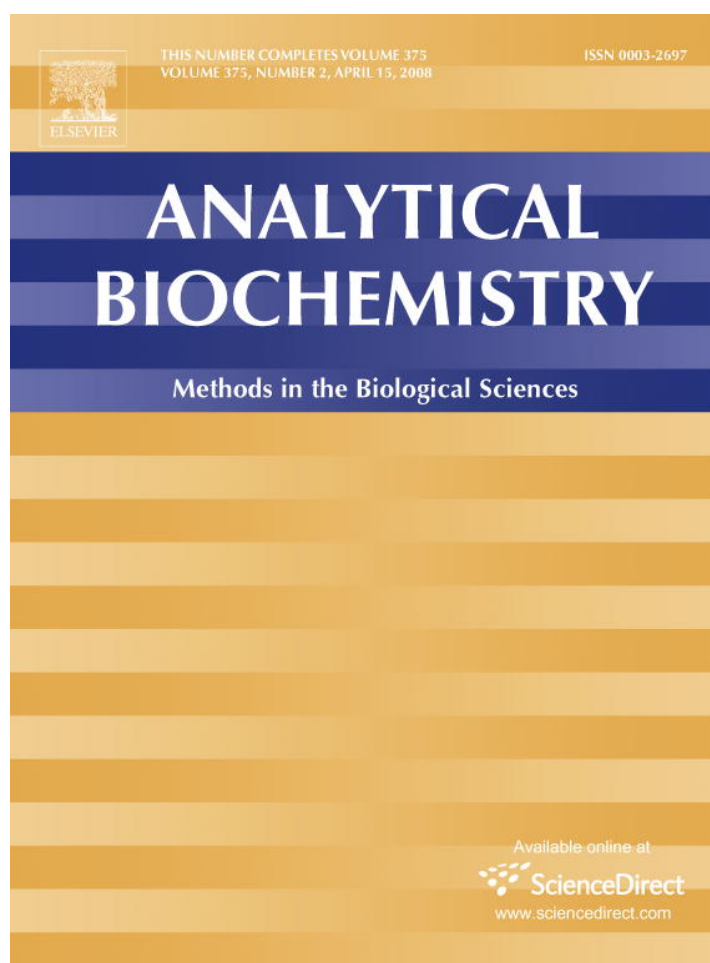


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Isotope-coded, iodoacetamide-based reagent to determine individual cysteine pK_a values by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

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Abstract

Cysteine reactivity in enzymes is imparted to a large extent by the stabilization of the deprotonated form of the reduced cysteine (i.e., the thiolate) within the active site. Although this is likely to be an important chemical attribute of many thiol-based enzymes, including cysteine-dependent peroxidases (peroxiredoxins) and proteases, only relatively few pK_a values have been determined experimentally. Presented here is a new technique for determining the pK_a value of cysteine residues through quantitative mass spectrometry following chemical modification with an iodoacetamide-based reagent over a range of pH buffers. This isotope-coded reagent, *N*-phenyl iodoacetamide (iodoacetanilide), is readily prepared in deuterated (d_5) and protiated (d_0) versions and is more reactive toward free cysteine than is iodoacetamide. Using this approach, the pK_a values for the two cysteine residues in *Escherichia coli* thioredoxin were determined to be 6.5 and greater than 10.0, in good agreement with previous reports using chemical modification approaches. This technique allows the pK_a of specific cysteine residues to be determined in a clear, fast, and simple manner and, because cysteine residues on separate tryptic peptides are measured separately, is not complicated by the presence of multiple cysteines within the protein of interest.

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Keywords: Thioredoxin; Iodoacetanilide; Thiolate; Chemical modification

Reactive cysteine residues in proteins typically are stabilized to at least some degree in their deprotonated thiolate form (i.e., with the pK_a of the thiol lowered from an unperturbed value of ~ 8.7) and are important players in a variety of enzymes catalyzing redox and hydrolytic reactions through an initial nucleophilic attack on substrate [1,2]. Unfortunately, pK_a values for catalytically important cysteine residues have been determined in only a small number of cases relative to the large number of representatives for which knowledge of this value would be of biological and chemical significance.

Methods for determining pK_a values of cysteinyl residues in proteins typically involve the measurement of a change associated with the varying thiol/thiolate content of the protein of interest exposed to buffers over a range of pH values. Changes associated with alteration of the protonation state of a cysteine include absorbance at 240 nm where the thiolate exhibits a modestly higher absorbance than does the thiol [3,4], nuclear magnetic resonance (NMR)² chemical shift changes associated

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² Abbreviations used: NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; Trx, thioredoxin; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ICAT, isotope coded affinity tag; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TCEP, tris(2-carboxyethyl)phosphine; DCC, dicyclohexylcarbodiimide; FPLC, fast protein liquid chromatography.

with protonation/deprotonation [5–7], and changes in rates of reaction of the cysteine of interest with electrophilic chemical modifying agents where the more nucleophilic thiolate anion is reactive but the protonated thiol is not [8–11]. In the latter case, this approach involves monitoring the progress of the chemical modification over time at each pH value; iodoacetate and its uncharged counterpart, iodoacetamide, typically are used in such studies, and progress of the reaction is measured by various methods, including functional assays [11–13] and high-performance liquid chromatography (HPLC) to separate unmodified protein from singly or doubly modified protein [14]. Particularly well studied is the thioredoxin (Trx) family of proteins, where the more N terminal of the cysteinyl residues in the CXXC motif exhibits a pK_a value as low as 3.3 to 3.5 for the oxidizing protein DsbA [10,14] or as high as 6.7 to 7.5 for the highly reducing thioredoxins [5,7,15,16].

Considering methods available for pK_a analyses, we sought to develop an approach to cysteinyl pK_a determination based on iodoacetamide modification that takes advantage of peptide analyses by quantitative mass spectrometry (MS). Our test protein for these studies was Trx from *Escherichia coli* for which chemical modification data with iodoacetamide had been reported previously [11,14]. With the approach introduced here, proteolytic digestion and matrix-assisted laser desorption ionization time-of-flight (MALDI–TOF) MS of proteins is used to monitor the extent of reaction of specific cysteinyl residues at various time points, an approach that requires very little protein and accurately maps the modification(s) to one or more specific peptides. The iodoacetamide derivative used here can be readily synthesized in two “isotope-coded” forms, incorporating no deuterium (d_0) or five deuterium atoms (d_5) into the *N*-phenyl iodoacetamide (iodoacetanilide) reagent to distinguish the products, similar to the original isotope coded affinity tag (ICAT) technique introduced by Aebersold and coworkers [17]. To provide the data needed to evaluate pK_a by chemical modification, a fully or partially modified d_5 -iodoacetanilide-labeled version of the protein is added in constant amounts to each quenched test sample taken over a time course of d_0 -iodoacetanilide reaction, tryptic digests are analyzed by MALDI–TOF MS for their ratio of light to heavy modified peptide to monitor reaction progress, and the modification reaction is evaluated over a range of pH values. With this approach, multiple cysteinyl sites on the same or separate peptides in a digest can be monitored simultaneously, obviating the need to study mutant proteins where one or more target cysteine residues have been replaced by serine or alanine. Thus, this MS-based approach provides a readily synthesized tool and a new analytical technique for conducting pK_a studies of cysteine-containing proteins of interest.

Materials and methods

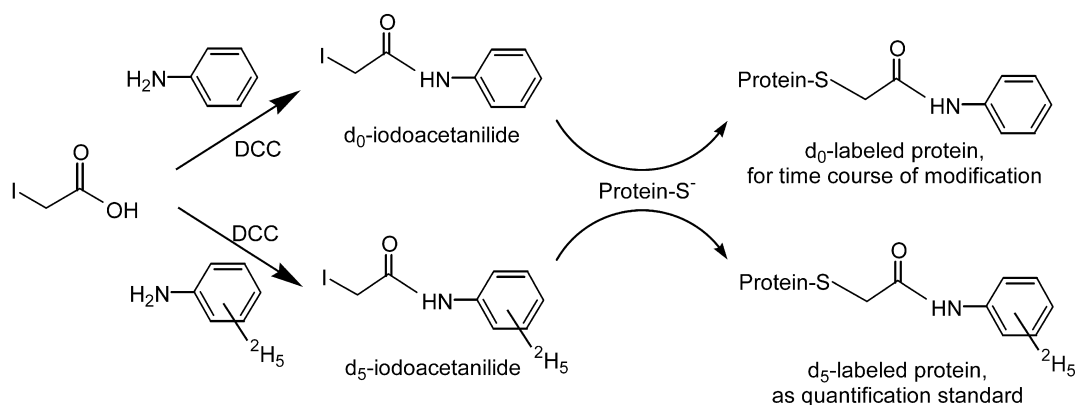
Materials

Trichloroacetic acid (TCA), dihydroxybenzoic acid, dibasic sodium phosphate, boric acid, ammonium sulfate, sodium citrate, 2-mercaptoethanol, aniline, iodoacetic acid, and iodoacetamide were obtained from Sigma–Aldrich. Ammonium bicarbonate, cysteine, and ethylenediaminetetraacetic acid (EDTA) were obtained from Research Organics. Dithiothreitol was purchased from Anatrace. Acetonitrile and dimethyl sulfoxide were purchased from Fisher. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) and sinapic acid were obtained from Fluka. Trifluoroacetic acid and tris(2-carboxyethyl)phosphine (TCEP) were obtained from Pierce Chemical. NADPH was obtained from Roche, ether was obtained from Mallinckrodt, and perdeuterated aniline was obtained from Cambridge Isotope Laboratories.

Iodoacetanilide (also denoted as d_0 -iodoacetanilide) was prepared from aniline and iodoacetic acid using dicyclohexylcarbodiimide (DCC) coupling (Scheme 1) as described previously [18]. Substitution of perdeuterated aniline for aniline in this procedure yields d_5 -iodoacetanilide. *E. coli* Trx reductase was expressed and purified as described previously [19,20]. *E. coli* Trx was expressed from *E. coli* strain CHW170 [21], which was a gift from Charles H. Williams at the University of Michigan (this is a wild-type *E. coli* strain transformed with an expression plasmid with wild-type Trx under control of a temperature-sensitive λ -promoter). Trx was purified as described previously [22] with the exception that all chromatography was conducted by fast protein liquid chromatography (FPLC) at 4 °C, a 75 ml Q-Sepharose HP column was used instead of a 200 ml column, and a 250 ml Suparose 12 prep-grade FPLC column was used instead of a Sephadex G50 size exclusion column. Pure protein was concentrated to 10 mg/ml in 20 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.6 and was stored in aliquots at –80 °C.

Comparison of rates of reaction of iodoacetamide and iodoacetanilide with free cysteine and with reduced Trx

For analysis of reaction rates of each reagent with free cysteine, reaction mixtures included 50 μ M cysteine in 5 ml of BPAGE buffer (10 mM sodium phosphate, 10 mM boric acid, 10 mM sodium citrate, 1 mM EDTA, and 100 mM ammonium sulfate at pH 7.0), to which iodoacetamide (0.25–2 mM) or iodoacetanilide (0.25–1 mM) (from a 200 mM stock prepared in dimethyl sulfoxide) was added and incubated at room temperature. An aliquot (0.3 ml) was removed at each time point, and the content of free thiols was determined by the addition of 0.2 ml of a solution containing 750 μ M DTNB (from a 50 mM stock in dimethyl sulfoxide) and spectral monitoring (on an Agilent HP8453 diode array spectrophotometer) of the



Scheme 1. Generation of isotope-coded iodoacetanilide using DCC coupling of iodoacetate with normal or perdeuterated aniline. Protein thiolates were then modified with d₀-iodoacetanilide over a time course or, as a separate quantification standard, with d₅-iodoacetanilide for further analyses as described in the text.

appearance of the colored product, 2-nitro-5-thiobenzoate ($\epsilon_{412} = 14,150 \text{ M}^{-1} \text{ cm}^{-1}$) [23]. The reaction time point was taken as the moment the DTNB was added to the cuvette, and all values were corrected for A_{412} observed when DTNB was added to buffer alone. The reaction rate monitored by loss of thiol content was determined by direct fit to a single exponential equation. Pseudo-first-order rate constants obtained in this way were then replotted versus reagent concentration to obtain the second-order rate constants for modification of cysteine at pH 7.0.

For evaluation of the reactivity of each of these reagents with a test protein, *E. coli* Trx was first reduced by incubation with dithiothreitol (20 mM final concentration) at 25 °C for 10 to 20 min. Dithiothreitol was removed and the protein was exchanged into BPAGE buffer at pH 7.0 by passing it through a PD-10 size exclusion column (GE Healthcare). Protein concentration in the pooled fractions was determined using an extinction coefficient at 280 nm of $13,700 \text{ M}^{-1} \text{ cm}^{-1}$ [24]. The solution was brought to a final concentration of 8 μM Trx in 7.5 ml, and the reaction was started by the addition of 80 μM iodoacetamide or iodoacetanilide. A portion of the reaction mixture (0.48 ml) was removed at each time point, and the content of free thiols was determined as described above for cysteine except that 5 μl of a 30 mM stock of DTNB in dimethyl sulfoxide was added. Apparent first-order rate constants were again obtained by fitting the time course data to a single exponential equation.

Quantitative analysis by MS of labeled cysteine-containing peptides

E. coli Trx (250 μM) in 25 mM potassium phosphate buffer at pH 7.0 was incubated with 1 μM Trx reductase and 500 μM NADPH at room temperature for 10 to 15 min to fully reduce the Trx and then was diluted 50-fold into BPAGE buffer at pH 7.0. To this solution was added 50 μM of either d₀-iodoacetanilide or d₅-iodoacetanilide, and the reaction was allowed to continue at 23 °C for varying times before being quenched with 2-mercaptoethanol. For

each labeling agent, two samples were prepared; one reaction mixture was incubated for 30 min to obtain exclusively singly labeled Trx, and a separate reaction mixture for each was incubated for 25 to 40 h to obtain the doubly labeled protein. The amount and nature of labeled protein in each reaction mixture were determined by MALDI-TOF MS analysis of the intact proteins. For each set (singly and doubly labeled), the labeled proteins were combined in known ratios of d₅-labeled (1.25 nmol) and d₀-labeled (0–12.5 nmol) proteins, assuming that all of the protein in each sample was found in the desired labeled state. To concentrate and exchange the samples into a buffer suitable for trypsin digestion and MALDI-TOF MS analysis, each 0.5 ml sample was precipitated by the addition of 50 μl of 100% (w/v) TCA for 20 min at –20 °C, followed by centrifugation at 14,000g for 15 min at 4 °C. The protein pellet was washed with 0.5 ml of 1:1 ethanol/ether, recentrifuged, and resuspended in 50 μl of 36 mM ammonium bicarbonate, 1 mM CaCl₂, and 10% acetonitrile for digestion with 1.25 μg trypsin (16 h at 37 °C). Each sample was mixed in a 1:1 ratio with matrix (0.01 mg sinapic acid in 1 ml of 50% acetonitrile and 0.3% trifluoroacetic acid) and spotted onto a MALDI target plate. The tryptic peptide of interest from Trx contains both cysteinyl residues (ADGAILVDFWAEWCGPCK, 1979.89 Da unmodified), gaining 134.06 Da for each modification by iodoacetanilide. Following sample preparation and application to the target plate, the ratio of the heavy (d₅) and light (d₀) modified peptides in each sample was determined experimentally by measuring the relative intensities of the mono-isotopic peaks for the singly labeled peptides (at 2114 and 2119 Da for the d₀- and d₅-labeled peptides, respectively) and for the doubly labeled peptides (with alkylation of both cysteinyl residues in Trx, at 2248 and 2258 Da for the d₀- and d₅-labeled peptides, respectively) using a Bruker Daltonics MALDI-TOF mass spectrometer.

Generation of a heavy (d₅-labeled) Trx standard

d₅-Iodoacetanilide-labeled Trx was generated by incubating 2.8 mM d₅-iodoacetanilide with 0.29 mM Trx and

0.42 mM TCEP in 25 mM potassium phosphate buffer containing 1 mM EDTA at pH 7.6 for 4 h at room temperature in the presence or absence of 4 M guanidine-HCl. The labeling reactions were quenched by the addition of 100 mM 2-mercaptoethanol, and the protein samples were exchanged into 25 mM phosphate buffer containing 1 mM EDTA at pH 7.0, using multiple cycles of centrifugation and redilution with a Microcon-30 ultrafiltration device (Millipore). After establishing the content of singly and doubly labeled protein in each sample by MALDI-TOF MS (with the doubly labeled product predominating in the sample to which the guanidine had been added), the samples were combined to ensure the presence of approximately equal amounts of both singly and doubly labeled protein and aliquots were frozen at -80°C until needed.

Determination of the pK_a value for reduced Trx

E. coli Trx was reduced using the physiological reductants (NADPH and Trx reductase) as described above. The sample was then brought to a final concentration of 5.0 μM Trx in 9 ml of BPACE buffer adjusted to the desired pH with either ammonium hydroxide or 20% sulfuric acid. The reaction was started by the addition of 50 μM d₀-iodoacetanilide, and the samples were incubated at 23 $^{\circ}\text{C}$ in a temperature-controlled water bath. The final pH of each reaction mixture was measured. At each time point, 0.5 ml of the solution (2.5 nmol of Trx) was removed and quenched with a final concentration of 250 mM 2-mercaptoethanol and 1.25 nmol of the d₅-labeled Trx standard was added. As described above for the peptide quantitation, samples were TCA precipitated and exchanged into 36 mM ammonium bicarbonate, 1 mM CaCl₂, and 10% acetonitrile and were incubated with 1.25 μg trypsin for 16 h at 37 $^{\circ}\text{C}$.

The extent of the Trx reaction with d₀-iodoacetanilide at each time point was measured by MALDI-TOF MS analysis. Samples were mixed with sinapic acid and spotted onto a MALDI target plate as described above. Data were collected in positive ion mode using the AutoRun feature, and each sample was analyzed three times. To determine the relative ratio of the singly labeled Trx peptide, the intensity of the monoisotopic peak of the light derivative (2114 Da) was divided by the intensity of the monoisotopic peak of the heavy derivative (2119 Da). The same procedure was used for the doubly labeled peaks at 2247 and 2257 Da for the light and heavy derivatives, respectively.

Rates of reaction were determined between pH 4.0 and pH 10.6 for Trx reacting with one or two molecules of iodoacetanilide according to the consecutive irreversible kinetic model of $A \rightarrow B \rightarrow C$, where A is unlabeled peptide, B is singly labeled peptide, C is doubly labeled peptide, and k_1 and k_2 are the rate constants for each step ($A \rightarrow B$ and $B \rightarrow C$, respectively) [25]. Data for the generation of B over time t were plotted and fit directly to Eq. (1) using KaleidaGraph 4.0 (Synergy Software):

$$[B]_t = \frac{[A]_0 k_1}{k_2 - k_1} [\exp(-k_1 t) - \exp(-k_2 t)]. \quad (1)$$

The rate of appearance of species C can be described by Eq. (2):

$$[C]_t = [A]_0 \left\{ 1 + \frac{1}{k_1 - k_2} [k_2 \exp(-k_1 t) - k_1 \exp(-k_2 t)] \right\}. \quad (2)$$

Because $k_1 \gg k_2$ at all pH values, the rate equation for the less reactive cysteine ($B \rightarrow C$) was simplified to a single exponential equation (Eq. (3)):

$$[C]_t = [A]_0 [1 - \exp(-k_2 t)]. \quad (3)$$

The pK_a values for the fast- and slow-reacting cysteine residues were then calculated by direct fits of each observed rate constant to Eq. (4) [26], where y is the observed rate constant (k_1 or k_2) at a given x (pH), k_{HA} is the limiting rate constant for the reaction at low pH (the protonated thiol form), and k_{A^-} is the limiting rate constant for the reaction at high pH (the deprotonated thiolate form):

$$k_{\text{HA}} \text{ and } k_{\text{A}^-} \text{ were allowed to vary during the fits.} \\ y = \frac{k_{\text{A}^-} \times 10^x + (k_{\text{HA}} \times 10^{\text{pK}_a})}{10^x + 10^{\text{pK}_a}}. \quad (4)$$

Sequencing of the singly labeled Trx peptide

A sample containing singly labeled Trx was generated by incubating pre-reduced Trx with iodoacetanilide for 20 min in BPACE buffer at pH 6.0. As above, the sample was exchanged into 36 mM ammonium bicarbonate, 1 mM CaCl₂, and 10% acetonitrile and was digested with 1.25 μg trypsin for 16 h at 37 $^{\circ}\text{C}$. The sample was spotted onto a MALDI target using a matrix made up of saturated dihydroxybenzoic acid in 50:50 acetonitrile/water with 0.1% trifluoroacetic acid, and the sequence of the peptide corresponding to a single *N*-phenyl iodoacetamide label (2113.8 Da) was determined by the fragmentation pattern obtained from a 4700 Proteomic Analyzer MALDI-TOF-TOF MS (Applied Biosystems).

Results

Synthesis and testing of isotope-coded iodoacetamide derivatives

For development of an isotope-coded thiol reagent based on iodoacetamide that would allow for quantitative assessment of reaction progress by MALDI-TOF MS, we chose to derivatize iodoacetic acid with aniline using a simple, one-step synthetic procedure where either normal aniline or perdeuterated aniline was coupled to iodoacetamide to yield *N*-phenyl iodoacetamide (Scheme 1, d₀- or d₅-iodoacetanilide) [18]. Thus, on reaction with a thiol group, the products of the modification with these compounds differ by 5 atomic mass units, a difference that is sufficient to readily discriminate between the multiple iso-

topic peaks for the peptides labeled with d_0 -iodoacetanilide and those for the peptides labeled with d_5 -iodoacetanilide [27].

To assess the reactivity of iodoacetanilide with thiol groups compared with the commonly used iodoacetamide reagent, various concentrations of the alkylating agents (0.25–2 mM) were added to solutions of 50 μM cysteine at pH 7.0 and thiol contents were assessed at each time point using DTNB assays. Pseudo-first-order rate constants were then replotted versus reagent concentration to obtain the second-order rate constants for reaction (Fig. 1). By this measure, d_0 -iodoacetanilide is threefold more reactive toward cysteine (at $110 \text{ M}^{-1} \text{ min}^{-1}$) than is iodoacetamide ($36 \text{ M}^{-1} \text{ min}^{-1}$), indicating that the addition of the phenyl group to the amido nitrogen does not adversely affect the reactivity of this reagent toward thiol groups. Because the iodoacetanilide was to be used with Trx in the subsequent experiments and accessibility of sites of reaction could be more restricted in the protein than for cysteine, we also assessed the rate of reaction of 80 μM of each of the reagents toward 8 μM Trx at pH 7.0. Under these conditions, an approximately sevenfold higher rate of reaction was observed for d_0 -iodoacetanilide and Trx (1.70 min^{-1}) compared with iodoacetamide and Trx (0.24 min^{-1}) (data not shown), verifying that iodoacetanilide is both reactive and not adversely affected by any restriction in its access to the most reactive thiol group in Trx.

Evaluation of the applicability of d_0 - and d_5 -iodoacetanilide modification to quantification of peptides by MALDI–TOF MS

To confirm that these reagents could be used to provide quantitative data for MALDI–TOF MS analyses, four separate labeled Trx samples were generated with either d_0 -iodoacetanilide or d_5 -iodoacetanilide and incubated for

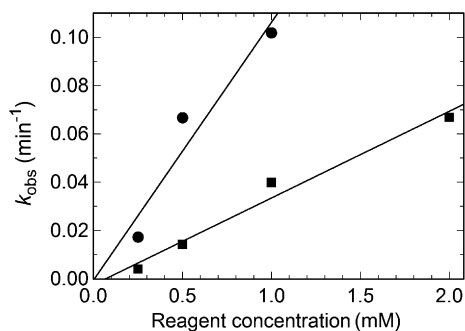


Fig. 1. Rate constants for free cysteine reacting with iodoacetanilide and iodoacetamide. Free cysteine (50 μM) was incubated with either iodoacetamide (squares) or iodoacetanilide (circles) at pH 7.0 and 23 $^{\circ}\text{C}$. For each time point at a given reagent concentration, a portion of the reaction mixture was removed and the free thiol content was determined by measuring the A_{412} on the addition of 300 μM DTNB. Values for the pseudo-first-order rate constants (k_{obs}) were determined by direct fit to a single exponential equation at each reagent concentration. Shown are the k_{obs} values plotted versus reagent concentration. The second-order rate constants were determined from the slope of the lines fit to the data in this plot.

either short (30 min) or long (25–40 h) times to obtain predominantly singly or doubly labeled protein, respectively. The differentially labeled pairs of protein samples were then mixed at various ratios, from 10-fold lower to 10-fold higher d_0 -labeled protein compared with the constant amount of d_5 -labeled protein. Samples subsequently were precipitated, digested with trypsin, and analyzed by MALDI–TOF MS. The relative signal intensities for pairs of peptide ions labeled with the d_0 - or d_5 -iodoacetanilide were determined and compared with the expected ratios. As shown in Fig. 2, expected ratios from 1:5 to 5:1 of the d_0 - versus d_5 -labeled peptides gave experimental intensity ratios that were linear when compared with the expected ratios. Although the fit was linear in both cases, the slope of the line was closer to 1 for singly labeled Trx peptide (1.09) than for doubly labeled Trx peptide (0.76). The variation from 1 for the slope of the doubly labeled samples can be explained by the fact that the reaction did not proceed to as full an extent for the d_0 reagent as for the d_5 -iodoacetanilide. This was because, despite the length of the reaction, it was difficult to consistently obtain a fully labeled Trx due to the slow reaction of Cys35; in this case, MALDI–TOF MS of the intact proteins showed a small but detectable peak of singly labeled Trx in the doubly labeled sample generated with the d_0 -iodoacetanilide. Although not ideal, this was not a significant problem because neither this experiment nor later experiments required an absolute concentration for the standard protein; rather, they required the consistent use of the same

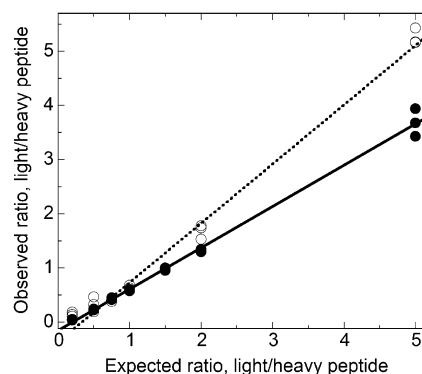


Fig. 2. Quantitative comparison of measured peptide ratios versus expected peptide ratios for d_0 - and d_5 -iodoacetanilide-labeled Trx. Prerduced *E. coli* Trx at pH 7.0 was incubated with either d_0 -iodoacetanilide (light) or d_5 -iodoacetanilide (heavy) in separate experiments for either 30 min or 25 to 40 h to obtain predominantly singly or doubly labeled protein, respectively. Each pair of heavy and light protein samples was mixed together in known ratios of the heavy (1.25 nmol) and light (0–12.5 nmol) proteins, precipitated with TCA, exchanged into 10% acetonitrile-containing ammonium bicarbonate buffer as described in Materials and methods, and digested with trypsin. The ratios of the heavy and light modified peptides were then determined for singly labeled (open circles) and doubly labeled (closed circles) Trx samples by measuring the relative peptide intensities using a Bruker Daltonics MALDI–TOF mass spectrometer. Plotted are the observed ratios versus the expected ratios (from 0.2 to 5.0) based on protein concentrations and the fit to a straight line.

standard. The good correlation between expected and measured ratios does, in any case, indicate the suitability of this technique for measuring reaction progress so long as a single sample of the d_5 -modified Trx is used as the quantification standard for all of the d_0 -modified samples to be analyzed at a given pH and the amount of d_0 -modified peptide is within 5-fold of the d_5 -modified peptide. For our purposes, a large batch of d_5 -iodoacetanilide-labeled Trx that contained both singly and doubly labeled protein was prepared, and this standard was aliquoted for use in subsequent experiments.

In separate experiments, comparisons were made of the tryptic cleavage products of modified Trx to determine whether or not this modification adversely affected cleavage by trypsin. For all modifications described in this article (doubly labeled, singly labeled, d_0 , and d_5) under the digestion conditions used, no peptides corresponding to a

missed cleavage site on either side of the labeled peptide were observed.

Evaluation of pK_a values for reduced Trx based on pH titration of the rates for iodoacetanilide modification

To determine the pK_a for the cysteinyl residues in Trx by our adapted chemical modification approach, reduced Trx was incubated with d_0 -iodoacetanilide in BPAGE buffer at various pH values and aliquots taken from the reaction mixtures at various time points were quenched with 2-mercaptoethanol. As alluded to above, a standard amount of Trx labeled with d_5 -iodoacetanilide containing both the doubly labeled and singly labeled products was added to each sample prior to buffer exchange and trypsin digestion. The ratio of light peptide (d_0 labeled) to heavy peptide (d_5 labeled) was calculated from the intensity val-

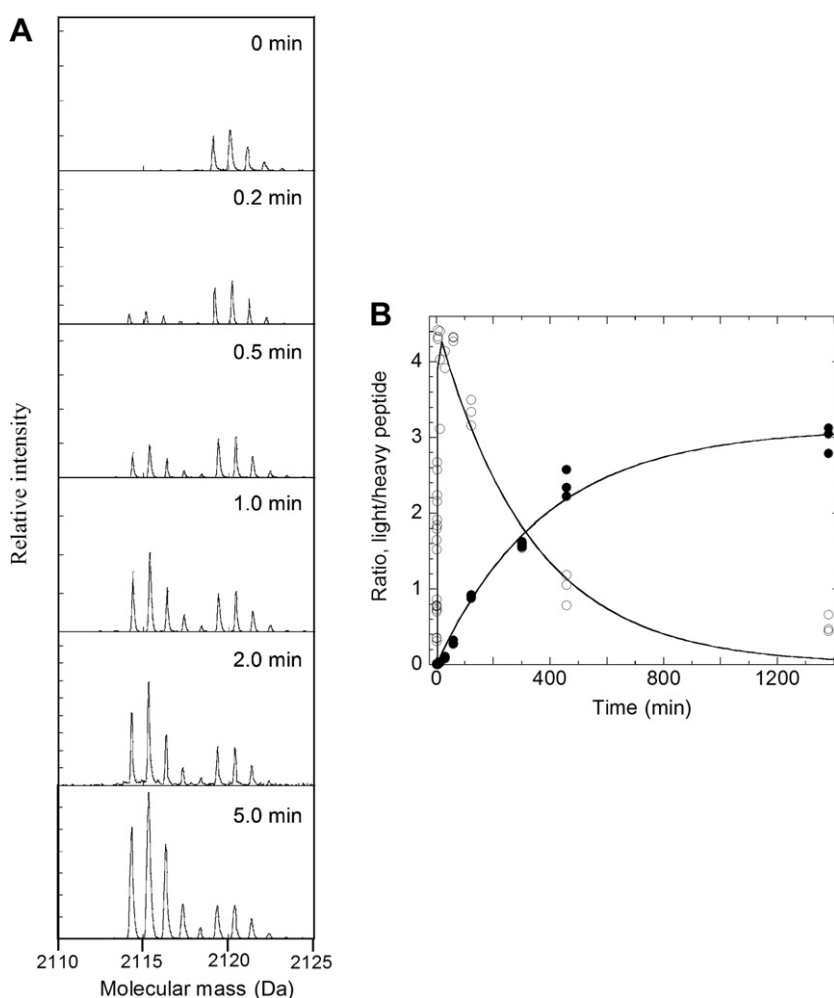


Fig. 3. MALDI-TOF MS data and fits to monitor the rates of reaction of iodoacetanilide with Trx. The extent of iodoacetanilide reaction with Trx was determined after trypsin digestion by analyzing peak intensities of the cysteine-containing peptide using a Bruker Daltonics MALDI-TOF mass spectrometer. Panel A shows sample data for the appearance of the singly d_0 -labeled peptide over time at pH 8.0. The ratio of peptide labeled with the d_0 -iodoacetanilide to that of the d_5 -labeled standard, which increased with time of reaction, was determined by dividing the intensity of the monoisotopic peak for the light peptide (~ 2114 Da) by the corresponding intensity of the monoisotopic peak for the heavy peptide (~ 2119 Da). As shown in panel B, the ratios for the singly labeled (2114 or 2119 Da, open circles) and doubly labeled (2247 or 2257 Da, closed circles) peptides were plotted as a function of time. Fits of the data to a kinetic model of $A \rightarrow B \rightarrow C$, where A is unlabeled peptide, B is singly labeled peptide (open circles), and C is doubly labeled peptide (closed circles) as shown by the lines, were carried out using Eqs. (1) and (3) as described in Materials and methods.

ues of the first monoisotopic peak from each (Fig. 3A). As shown in Fig. 3B, one cysteine reacts much more quickly than the other at all pH values (especially below pH 10), an observation that agrees with previous studies showing that Cys32 reacts much more quickly than Cys35 [11]. The irreversible reaction fits to an $A \rightarrow B \rightarrow C$ model, where A is unlabeled peptide, B is singly labeled peptide, and C is doubly labeled peptide and where the first step is fast and the second step is slow. As described in Materials and Methods, the difference in rates allows the appearance of C to be fit to a single exponential equation (Eq. (3)), whereas the appearance of B over time was fit to Eq. (1) (Fig. 3B). The experimentally determined rate con-

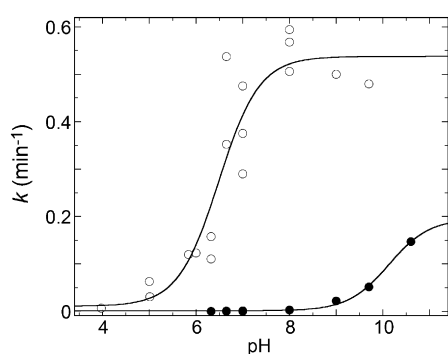


Fig. 4. Apparent pK_a determination for the fast- and slow-reacting cysteinyl residues of *E. coli* Trx. The pseudo-first-order rate constants for the singly labeled (k_1 , open circles) and doubly labeled (k_2 , closed circles) peptides of Trx were determined over the pH range of 4.0 to 10.6 as described in Materials and methods and plotted as a function of pH. Data were fit to Eq. (4) as described, allowing the pK_a value for each, as well as the rate constants for the upper and lower plateaus, to vary. Final results gave a pK_a of 6.49 ± 0.18 and an upper plateau of 0.537 ± 0.039 for the fast-reacting cysteine (identified by subsequent tandem MS analysis as Cys32) and a pK_a of 10.13 ± 0.05 and an upper plateau of 0.196 ± 0.007 for the slow-reacting cysteine (Cys35). Both rates dropped to zero at low pH within experimental error (0.001 ± 0.048 and 0.0011 ± 0.0008 , respectively).

stants for each step at various pH values from 4.0 to 10.6 were then plotted versus pH to determine the pK_a value for each cysteine (Fig. 4 and Eq. (4)). Using this approach, the calculated pK_a for the most reactive cysteine was 6.49 ± 0.18 . For the less reactive cysteine, the exact pK_a value was too high to be determined given the very limited data above pH 10.0, but the value obtained from the fit was 10.13 ± 0.05 (Fig. 4).

Tandem MS for identification of the fast-reacting cysteine residue in Trx

Previous studies have indicated that Cys32 reacts much more quickly than Cys35 [11]; therefore, based on data from the literature, the fast reaction with an observed pK_a of approximately 6.5 can be attributed to the alkylation of Cys32, whereas the slow rate can be attributed to the alkylation of Cys35. To confirm that this is indeed the case in our experiments, the 2113.8-Da peptide from Trx that contained only a single acetanilide label was sequenced using tandem MS analysis with a MALDI-TOF-TOF instrument. The fragmentation spectra included peptide fragments that indicated the presence of the label at Cys32 of Trx, whereas none that supported a label at Cys35 was observed (Fig. 5).

Discussion

We have presented a new and sensitive MS-based method to determine the pK_a for cysteine residues in proteins using a combination of the well-established technique of following chemical modification rates over a range of pH as a measure of thiol pK_a with a simple isotopic labeling of an iodoacetamide derivative to enable readout of the data by MS. The reagents required for this method are readily synthesized in reasonable quantities from commercially available precursors. In the original report of the

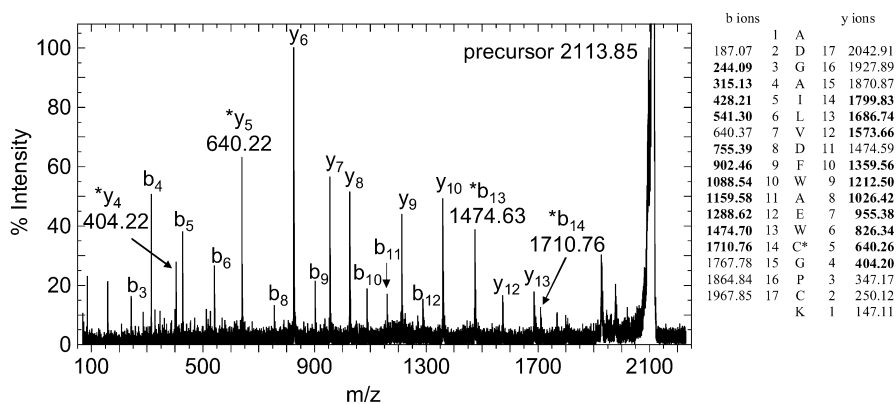


Fig. 5. MALDI-TOF-TOF spectrum of the singly, d_0 -iodoacetanilide-labeled peptide of Trx. The protein adduct was digested with trypsin to generate an 18-residue cysteine-containing peptide. The digest was spotted onto the MALDI target using dihydroxybenzoic acid as the matrix, and the labeled peptide (m/z 2113.85) was selected for fragmentation. The spectrum shown is the sum of 10 separate measurements. Cleavage of the amide bond results in N-terminal fragments designated as b and C-terminal fragments designated as y. The masses of both sets of ions clearly identify the d_0 -acetanilide linked covalently to Cys32 ($b_{14} - b_{13} = y_5 - y_4 = m/z$ 236.06). Peptides in the table marked in bold are found in the spectrum, and asterisks indicate peaks in the spectrum that demonstrate labeling on Cys32. No peaks consistent with labeling on Cys35 were observed.

synthesis of d₅-iodoacetanilide, the deuterated label was incorporated into proteins for neutron scattering studies [18]. In later modifications, ¹³C-labeled iodoacetanilide was generated for quantification of peptides and proteins using MS as a potential alternative to ICAT technology [27,28]. With the current focus on pK_a analysis, these reagents allow for the use of minimal amounts of protein given that MALDI–TOF MS is capable of detecting protein amounts considerably lower than those used in this study. Although altered chromatographic separation of peptides possessing deuterium labels can adversely affect MS-based quantification and has led to a preference for ¹³C or ¹⁵N labels over ²H in isotope-coded tags [29,30], the current method requires no separation of peptides before MALDI–TOF analysis and is unaffected by this limitation. If analysis of a complex mixture of peptides requiring chromatographic separation prior to MS analysis were to be carried out, ¹³C-labeled iodoacetanilide would be the better choice as the modifying agent [28].

With previous approaches using iodoacetamide reactivity to measure the pK_a of cysteine residues, subsequent assays for modification were unable to distinguish between or separately evaluate multiple cysteines in the same protein. By using tryptic digestion and MALDI–TOF MS as introduced here, the extent of reactivity with each cysteine residue can be monitored directly without the need for mutagenesis to distinguish cysteinyl residues. This method can be easily adapted to other less well-characterized proteins that contain cysteine residues, including those for which the physiological substrate is unknown. Because each cysteine residue is monitored independently, this technique can easily accommodate situations where the various cysteines in the protein react at very different rates and exhibit quite different pK_a values. In most cases, the protease, or even multiple proteases, used for the digestion can be optimized to ensure that each cysteine in the protein is located on a separate peptide of appropriate size. Although this is not readily accomplished in the case of *E. coli* Trx, the two rates are easily distinguished. Cys32 has been shown to be solvent accessible, whereas Cys35 is buried and protected from solvent [31]. This rate difference is further enhanced at pH values below 8.0 due to the differences in the pK_a values between Cys32 and Cys35. In addition, as we have done here, the identity of the fast- and slow-reacting cysteinyl residues (if they are on the same peptide) can easily be confirmed by using tandem MS analysis to sequence the modified peptides. Although we did not observe any missed cleavage sites in Trx even after modification, the presence of missed cleavage sites for other modified peptides will need to be determined experimentally for any other proteins and proteases used, and if any are present, their ratios will need to be accounted for during data analysis.

To confirm that our method gives results comparable to those with other chemical modification approaches, we tested it with *E. coli* Trx. This protein contains two cysteine residues that are critical for its activity, and the pK_a values

for these residues were determined by chemical modification with iodoacetamide in several instances, allowing us to easily compare our results with those of previous determinations. The pK_a values presented here of 6.49 ± 0.18 for Cys32 and greater than 10 for Cys35 closely agree with previously published pK_a values using iodoacetamide and iodoacetic acid (6.7 or 7.1 for Cys32 and greater than 9.0 for Cys35) [11,14]. Thus, we now have an MS-based technique for evaluating the pK_a values of specific cysteine residues that will be widely applicable and is not complicated by the presence of multiple cysteines within the protein of interest.

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