Estimating the Extinction Coefficient for a Protein

Felix F. Vajdos

University Undergraduate Fellow, 1990-91

Texas A&M University

Department of Biochemistry

Approved

Fellows Advisor C. N. Pace

Honors Director D. J. Kyle

ABSTRACT: A new method has been developed for estimating the extinction coefficient for a protein. The only information that is needed is the amino acid composition of the protein, and this is readily available if the gene coding for the protein has been sequenced. This method will allow extinction coefficients to estimated with an accuracy of better than 4% for most proteins. This will allow much more quantitative studies than had been possible in the past with the many proteins that can be obtained in only small amounts. A nonlinear least squares analysis was used to calculate the "best" extinction coefficients over the wavelength range 272 nanometers (nm) to 286 nm for the chromophores tyrosine (Tyr), tryptophan (Trp) and cystine (-S-S-). These values were then used to estimate the extinction coefficients for 17 proteins, yielding estimates that were on average within 3.4% of the experimental values. For comparison, the extinction coefficients for the model compounds N-acetyl-Tyr-ethyl ester (N-Ac-Tyr-OEE), N-acetyl-Trp-ethyl ester (N-Ac-Trp-OEE), and oxidized glutathione were determined over the same wavelength range in the following solvents: water, 6 M guanidinium hydrochloride (GdnHCl), 8 M urea, 1-propanol, and formamide. It was found that Trp, Tyr, and -S-S- in proteins resemble these model compounds in 6 M GdnHCl and 8 M urea rather than in water or in the more nonpolar solvents 1-propanol and formamide. The estimates for protein extinction coefficients based on model compounds in 6 M GdnHCl and 8 M urea were on average within 3.8% and 3.9%, respectively, of the experimental values.

Introduction

One of the most important tasks in any chemical experiment is the accurate determination of concentrations. This task is no less important in protein chemistry and enzymology than in other disciplines, but can be considerably more difficult in these fields. Thus, a great deal of attention has been paid to the problem of accurate determination of protein concentrations (Lowry, 1951; Gill & von Hippel, 1989; Nozaki, 1986; Kupke & Dorrier, 1978).

One of the most sensitive methods of protein concentration determination developed was the Lowry method (Lowry, et al, 1951). This method is based upon the earlier Biuret assay (coordination of alkaline copper with peptide bonds), plus a second reaction (reduction of phosphomolybdate-phosphotungstate by tyrosine and tryptophan residues) to enhance its sensitivity. Despite the fact that the Lowry method is sensitive to quantities of protein as small as 5 micrograms (μg), several factors limit its use: 1) the reaction is time consuming (40-60 min) and 2) the amount of color development varies because of the different reaction stoichiometries between proteins (for both the peptide bond-alkaline Cu2+ coordination reaction and the phosphomolybdate-phosphotungstate reduction by tyrosine and tryptophan residues) (Boyer, 1986). Many colorimetric methods of protein concentration determination have been developed since Oliver Lowry published his method, but all of them have problems similar to those encountered

with the Lowry method. It should be noted that in any colorimetric assay, many commonly used laboratory reagents may interfere with color development, hindering accurate measurements and further limiting their use. Thus, while being extremely sensitive, colorimetric methods of protein concentration determination have many drawbacks, and their use is limited to relative concentration measurements.

A very accurate, sensitive, and fast method for concentration determination is the absorption of ultraviolet (UV) light by the protein molecule itself, commonly known as the A_{280} method (because most absorbance measurements are made at or near 280 nanometers (nm)). This method is based upon the Beer-Lambert law, which relates concentration and light-path length to a unitless quantity known as the absorbance of the solution, A. The absorbance, which can vary from 0 to infinity, is related to the concentration of solute molecules and path length by a constant, ϵ, which has units of $concentration^{-1}length^{-1}$. ϵ, or the extinction coefficient, is a solvent-dependent and wavelength-dependent term, and is equal to the slope of a plot of A versus concentration, provided the solution obeys the Beer-Lambert law. For most proteins in the micro- to milli- molar concentration range, the Beer-Lambert law is valid.

The extinction coefficient can be thought of as the absorbance of a one molar solution of the solute of interest with a 1 centimeter (cm) path length. Using this as a basis, and the fact that absorbencies are additive, one can represent the molar

extinction coefficient for a multichromophore particle such as a protein as the linear combination of molar extinction coefficients for chromophores within the particle. Thus,

$$\epsilon_{tot} = \sum_i \epsilon_i \times n_i \qquad (1)$$

where ϵ_{tot} is the total molar extinction coefficient, ϵ_i represents the molar extinction coefficient for a chromophore i, and n_i is the number of chromophore i per molecule of protein. For proteins, the chromophores of importance in the UV region of the electromagnetic spectrum are tyrosine (Tyr), tryptophan (Trp), phenylalanine (Phe), and disulfide bonds, or cystine (-S-S-). In the wavelength region 270 nm to 290 nm, however, only Tyr, Trp, and -S-S- have significant absorbencies, and the contribution to the total absorbance by Phe is negligible (Wetlaufer, 1962). Thus, in principle at least, one can estimate the molar extinction coefficient for a protein at 280 nm by simply summing the contributions from three of its component chromophores, as in (1). Note that equation (1) assumes that each chromophore absorbs light independently of all others, and that all n of chromophore i have identical extinction coefficients.

ϵ varies with solvent because of the interactions between chromophore and solvent. For example, when a chromophore is transferred from water to a solvent such as 1-propanol,the absorbance spectrum is generally shifted slightly (1-2 nm) to longer wavelengths and the intensity of the absorption is

increased, an effect presumably due to a change in the energy and probability of electronic transitions (Yanari and Bovey, 1960). This wavelength-intensity shift is observed when a protein unfolds, thereby "transferring" its chromophores from the hydrophobic interior of the protein to the aqueous environment. This change in the spectra of native proteins relative to their unfolded, or denatured, forms is enhanced even further by the "rigid matrix" effect of the protein interior on the chromophores. This effect has been observed in a variety of experiments, and results in a difference between what would be "expected" in an absorption spectrum based on model compound data and what is actually observed, presumably because of the fact that the number of conformations which the polypeptide (including chromophores) can assume is highly restricted relative to the same component structures free in solution, thereby refining the vibrational fine structure (Beaven and Holiday, 1952), shifting the absorption spectra to longer wavelengths (Yanari and Bovey, 1960; Edelhoch, 1967), and reducing spectral broadening (Brandts and Kaplan, 1973). Thus, the absorbance spectrum of a protein is a complex function of solvent composition and matrix fluidity, and for these reasons is only approximated by using model systems.

One of the first things learned about a protein is its amino acid sequence, and this is usually determined from sequencing the the gene which codes for it. If one accurately knew the molar extinction coefficients for Tyr, Trp, and -S-S- within a native protein structure, one could estimate the total molar absorbance

for a native protein molecule. This is an important proposition for many researchers, who often deal with microgram amounts (or less) of protein, and who cannot spare the milligram amounts required for (and/or the time to go through the lengthy and tedious) protein dry weight concentration calibration of the A_{280} method.

Two approaches for estimating the extinction coefficients for Tyr, Trp, and -S-S- within the folded protein were used in this study. A nonlinear least squares analysis was done on the equation

$$\epsilon_{tot} = \epsilon_{tyr} N_{tyr} + \epsilon_{trp} N_{trp} + \epsilon_{SS} N_{ss} \qquad (2)$$

in order to determine the values of ϵ_{tyr}, ϵ_{trp}, and ϵ_{ss}, which minimized the sum of the squared residuals, S,

$$S = \sum_i (\epsilon_{tot} - \epsilon_{exp})^2 \qquad (3)$$

where ϵ_{exp} is the experimental molar extinction coefficient. For comparison, the model compounds n-acetyl-tyrosine-ethyl ester (N-Ac-Tyr-OEE), n-acetyl-tryptophan-ethyl ester (N-Ac-Trp-OEE), and oxidized glutathione (ox. glu.) were examined in water, 6M guanidinium hydrochloride (GdnHCl), 8M urea, 1-propanol, and formamide. Using similar model compounds in 6 M GdnHCl, Edelhoch (1967) qualitatively matched the absorbance spectrum of denatured ribonuclease A. Later, Gill and von Hippel (1989) used Edelhoch's data to accurately estimate the extinction coefficients for 19 proteins, with an average difference from experimental values of 5%. For this study, the n-acetylated derivatives of Tyr and Trp

were chosen to model the peptide bond at the α-amino group of the chromophore. Substitution at the α-carboxyl group apparently has little effect on the absorption spectrum (Edelhoch, 1967), and the ethyl esters were used for both amino acids to be experimentally consistent. Oxidized glutathione (2 molecules of γ-Glu-Cys-Gly joined by a disulfide bond at the Cys residues) was chosen as a model compound for -S-S-.

Materials and Methods

N-Ac-Tyr-OEE was purchased from American Tokyo Kasei, Inc. N-Ac-Trp-OEE, oxidized glutathione (Grade III), and formamide (ACS grade) were purchased from Sigma Chemical Co. Urea was Ultrapure from United States Biochemical Corp. Guanidinium Hydrochloride (Heico's Synthesized Extreme Purity) was purchased from Heico, Inc. 1-Propanol was purchased from Fisher Scientific. All water was double-distilled in an all-glass still.

For the model compounds, stock solutions of 50% propanol-50% water were prepared in concentrations sufficient to yield a 0.5 % propanol content in the solutions on which UV spectra were measured. This propanol content was assumed to have no perturbing effect on the absorbance spectra. All solvents were filtered prior to making solutions using a 0.22 um filter and a Millipore filtration apparatus. The UV spectrum from 350 nm to 250 nm were recorded for N-Ac-Tyr-OEE, N-Ac-Trp-OEE, and oxidized glutathione using a Cary Model 15 recording spectrophotometer. Matched Beckmann quartz cells (1 cm path length) were used, and were cleaned with concentrated nitric acid between experiments.

For the dry-weight determinations of protein concentration, a modified version of the method described by Kupke and Dorrier (1978) was used. Protein solutions were prepared by dissolving approximately 80-100 mg of protein in 10 mL of glass distilled water, and then clarified using a 0.2 um Acrodisc syringe filter. This solution was then dialyzed exhaustively at 4 C against four 1 L volumes of water. Weigh bottles (25 mm X 40 mm, 10 mL volume)

were purchased from Fisher Scientific, and were scrupulously washed between dry weight determinations, first with concentrated nitric acid to hydrolyze the dry protein residue, then with laboratory detergent and tap water, followed by rinsing with copious volumes of glass distilled water. The bottles were allowed to dry at 98 C for at least 8 hours, followed by at least 3 hours of cooling in a very clean, greaseless dessicator under house vacuum (approximately 22 inches Hg) before the bottle weight was measured on a Mettler H10 balance reading to 0.5 mg. Following this, exactly 2.00 mL of protein solution was placed in each weigh bottle using a pipette calibrated at 21 C with glass distilled water. The solutions were then dried 4-6 hours at 98 C in a conventional, convection oven, followed by 10-12 hours at 100 C in a Fisher Scientific model 280 vacuum oven under house vacuum. The samples were found to achieve constant weight after this amount of drying time. The bottles were removed from the vacuum oven using dry acid-washed tongs, placed in the dessicator and allowed to cool under house vacuum for at least 3 hours. The bottle+residue weights were then measured on the Mettler H10 balance, and the bottle weight was subtracted to give the protein residue weight. Protein concentrations (in moles per liter) were calculated by dividing the residue weight (in mg) by the volume (2.00 mL) and then multiplying by the molecular weight. Absorption spectra from 350 nm to 250 nm were recorded using the Cary Model 15 recording spectrophotometer described previously. It should be noted here that the author performed this dry weight procedure only for lysozyme, bovine serum albumin, ribonuclease A,

and ribonuclease T_1; the other protein extinction coefficients were determined by others in the same lab over the time period 1980-1989.

Nonlinear analysis of equation (2) was performed using NONLIN, a nonlinear parameter estimation package written by Michael L. Johnson, (650 Chapel Hill Road, Charlottesville, VA 22908; (804)-924-8607, (804)-973-3114). As nonlinear analysis methods lend themselves better to analyzing raw data sets, rather than averages, (Johnson & Frasier, 1985), the triplicate extinction coefficients were used for the proteins analyzed by the author.

Results

Model compound studies

The absorbance spectra of N-Ac-Tyr-OEE, N-Ac-Trp-OEE, and oxidized glutathione are shown in Figures 1, 2, and 3. For N-Ac-Tyr-OEE, (Figure 1) there is a distinct "red-shift", or shift toward longer wavelength, and a noticable increase in absorbance upon going from water to 1-propanol, with overlapping spectra for 6M GdnHCl and 8M urea, and also for 1-propanol and formamide. The spectra for N-Ac-Trp-OEE (Figure 2) do not show the same overlap, except for a slight overlap in 6M GdnHCl and 8M urea. The spectrum for N-Ac-Trp-OEE in formamide clearly does not overlap with that using 1-propanol as the solvent. This effect could be due to the fact that 1 mm path length cells were used for determining the spectrum in formamide, while 1 cm path length cells were used for all other spectra, but the fact that the same 1 mm cells were used to determine the N-Ac-Tyr-OEE spectrum in formamide casts some doubt upon this. In Figure 3 is shown the spectra for oxidized glutathione in water, 6M GdnHCl, and 8M urea. The spectra are not seen to overlap as in N-Ac-Tyr-OEE and N-Ac-Trp-OEE, but the general trend is still the same; that is, a red-shift upon moving from water to 8M urea to 6M GdnHCl, with a concomitant increase in absorbance. The spectra for oxidized glutathione in 1-propanol and formamide were not obtained due to the limited solubility of oxidized glutathione in these solvents.

Nonlinear parameter estimation

The extinction coefficients for Tyr, Trp, and -S-S- calculated

using the NONLIN program compare favorably with those obtained for the model compounds N-Ac-Tyr-OEE, N-Ac-Trp-OEE, and oxidized glutathione (see Tables 1-3, and Figure 4). In Figure 4, the absorption spectra (calculated and model compounds) for Tyr, Trp, and -S-S- are shown. The model compound spectra (dotted lines) are in the solvents 6 M GdnHCl and 8 M urea, and are indistinguishable except for Trp. The calculated spectra appear to be red-shifted with respect to the model compound spectra, and also seem to have greater absorbencies than the model compounds. The exception to this is Trp, the absorption of which exceeds the model compounds only at the long-wavelength end of the spectrum. A curious result of the nonlinear parameter analysis is the spectrum for -S-S- (see Fig. 4, S-S spectrum, dark line), which increases in absorbance as wavelength increases. Another curious result for -S-S- is that there is a dip in the spectrum which is not very obvious on the scale of Figure 4, but is apparent from the data in Table 1. The source of this dip, which does not appear with oxidized glutathione, is unknown, and might be an artifact of the nonlinear parameter estimation method. Because of the similarity between the calculated chromophore spectra and the model compound spectra in 6 M GdnHCl and 8 M urea, and because the model compounds in the other solvents studied clearly were either too high (N-Ac-Tyr-OEE in 1-propanol, for instance), only these chromophore extinction coefficients were used in estimating the protein extinction coefficients.

Estimation of molar extinction coefficients for proteins

The estimates of the extinction coefficients at 280 nm for 17 proteins are listed in Table 4. The estimates are based on the chromophore extinction coefficients obtained by nonlinear parameter estimation, model compounds in 6M GdnHCl, and model compounds in 8M urea. The experimental values listed were obtained by the dry weight concentration method described in **Materials and Methods.** Several proteins were included more than once in the data set upon which the nonlinear analysis was done, as recommended by Johnson and Frasier (1985). The differences between the estimated extinction coefficients and the experimental values are listed in Table 5. The calculated spectra for three representative proteins (lysozyme, RNase T_1, and RNase A) are compared with the experimental spectra in Figures 5 through 7. These three proteins were chosen for their differing Trp content (lysozyme has 6 trp, RNase T_1 has 1, and RNase A has 0), since the absorbance by Trp dominates the spectrum at this wavelength.

Discussion

As shown in Figures 5, 6, and 7, the estimates of the molar extinction coefficients from 272 nm to 286 nm for the three representative proteins based upon the calculated extinction coefficients for Tyr, Trp, and -S-S- match the experimental values much better than those based upon model compounds in either 6M GdnHCl or in 8M urea. There are several plausible explanations for this. First, as noted by Beaven and Holiday (1952), and later by Yanari and Bovey (1960), there is a noticeable red-shift in a chromophore's absorption spectrum when it is embedded in a semi-solid matrix. Since the model compounds studied were free in solution, this effect was not taken into account, and consequently estimates of a protein's extinction coefficient are likely to be in error. The chromophore extinction coefficients extracted from nonlinear analysis of equation (2) represent the "best" values for a chromophore within a folded protein, the structure of which has been suggested to be very solid-like in nature (Sandberg and Terwilliger, 1991). Second, it is plausible that a chromophore within a folded protein interacts to a greater extent with hydrophobic side chains of other residues than with the peptide backbone. If such is the case, then solvents such as GdnHCl and urea, which resemble the carbon backbone, might not be good solvents for modeling the interior of a folded protein. Perhaps the protein interior resembles some mixture of guanidine (or urea) and a hydrophobic liquid such as octanol. Whatever the reason, the nonlinear parameter estimation method offers a more accurate, if

less precise, method of estimating the extinction coefficient for a protein. This hypothesis is supported by the data in Table 5, in which are listed the deviations of estimated extinction coefficients from experimental extinction coefficients. The average deviations, listed at the bottom of the table, suggest that on average, the nonlinear regression method is slightly more accurate.

The nonlinear regression method does, however, tend to be less precise in estimating the extinction coefficient than corresponding model compound estimation methods, as shown by the errors in estimation given in Table 4. This results from the high error in the calculated chromophore extinction coefficients, contrasted with the relatively low error in the model chromophore extinction coefficients (Tables 1, 2, and 3). There are several reasons for this. First, in any nonlinear parameter estimation method it is imperative that the random errors in the data assume a Gaussian distribution and that no systematic error exists in the data (Johnson and Frasier, 1985). Experimental results for Rnase T_1 have yielded values for ϵ_{280} that range from 16943 $M^{-1}cm^{-1}$ to 18288 $M^{-1}cm^{-1}$. It is difficult to ascertain whether this wide range of values is due to changes in experimental conditions (i.e.-relative humidity, ambient temperature) or simply random error in measurement. A further consideration in this question is the effect of spectrophotometric error due to light scattering. All of the experimental values given in Table 4 calculated after correcting for light scattering using the Rayleigh formula,

$$K = C \times \lambda^{-4}$$

where K is the loss of light intensity due to scattering effects, C is an empirical constant, and λ is the wavelength. Beaven and Holiday (1952), however, have suggested that the exponent to which λ is raised may not always be equal to -4, and give several methods for experimentally determining the value of this exponent. They have also postulated that most of the variation in the literature for molar extinction coefficients for proteins might be due to errors in correcting for light scattering. Thus, part of the error in calculating the chromophore extinction coefficients is surely due to errors in the experimental extinction coefficients for the proteins in the data set. Second, in developing this method, it was assumed that the environments for Tyr, Trp, and -S-S- were generally the same for most globular proteins. Indeed, this may not be the case, as suggested by the large error in estimation for BPTI and insulin (Table 5). The extinction coefficients for these proteins are clearly modeled much more accurately by model compounds in either 6M GdnHCl or 8M urea, suggesting that the chromophores either are more exposed to the aqueous environment surrounding the protein molecule or have a greater degree of mobility than in other globular proteins (i.e.-they reside in a more fluid environment). This is an interesting possibility, and warrants further investigation.

In conclusion, chromophore extinction coefficients for the protein chromophores Tyr, Trp, and -S-S- can be determined directly from a knowledge of the chromophore content of a set of proteins

and the experimental extinction coefficients for those proteins. Furthermore, the extinction coefficient for a globular protein can be estimated very accurately by using these chromophore extinction coefficients and equation (1). Estimates based on model compounds in various solvent systems compare favorably with experimental data, but problems in finding a suitable solvent to accurately model the interior of a folded protein make this method less accurate than direct calculation of chromophore extinction coefficients. It is hoped that the method of nonlinear parameter estimation will be applicable to any globular protein of known amino acid and cystine content. Further studies on more proteins (to increase the size of the data set) should demonstrate the generality of this method.

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Table 1: Molar extinction coefficients for Tyr, Trp, and -S-S- measured by nonlinear parameter estimation of equation (2). Standard deviation is given in parentheses below each value.

λ	272	274	276	278	280	282	284	286
Tyr	1268 (84)	1360 (74)	1443 (67)	1453 (61)	1374 (57)	1287 (53)	1188 (67)	990 (82)
Trp	5116 (180)	5277 (159)	5300 (143)	5316 (128)	5468 (121)	5561 (115)	5380 (143)	4892 (176)
-S-S-	123 (103)	130 (91)	158 (82)	218 (74)	248 (70)	222 (66)	252 (82)	341 (100)

Table 2: Molar extinction coefficients for the model compounds N-Ac-Tyr-OEE (Tyr), N-Ac-Trp-OEE (Trp), and Oxidized Glutathione (O.G), measured using 6 M GdnHCl as solvent. The error in measurement is given in parentheses below each value.

λ	272	274	276	278	280	282	284	286
Tyr	1300 (10)	1410 (11)	1462 (11)	1388 (11)	1288 (10)	1245 (10)	1090 (9)	750 (6)
Trp	5260 (42)	5360 (42)	5407 (43)	5553 (44)	5680 (45)	5667 (45)	5347 (42)	4933 (40)
O.G.	193 (2)	174 (1)	158 (1)	140 (1)	124 (1)	110 (1)	96 (1)	84 (1)

Table 3: Molar extinction coefficients for the model compounds N-Ac-Tyr-OEE (Tyr), N-Ac-Trp-OEE (Trp), and Oxidized Glutathione (O.G), measured using 8 M Urea as solvent. The error in measurement is given in parentheses below each value.

λ	272	274	276	278	280	282	284	286
Tyr	1300 (10)	1400 (11)	1450 (11)	1388 (11)	1298 (10)	1242 (10)	1090 (9)	770 (7)
Trp	5253 (42)	5333 (42)	5387 (43)	5533 (44)	5633 (44)	5580 (44)	5233 (42)	4840 (40)
O.G.	178 (1)	162 (1)	145 (1)	130 (1)	115 (1)	102 (1)	89 (1)	78 (1)

Table 4: Estimates of and experimental values for molar extinction coefficients at 280 nm (ϵ_{280}) for the 17 proteins analyzed in this study. Numbers within parentheses beneath names represent the number of experimental values used, while numbers in parentheses beneath the values for ϵ_{280} represent the error in the estimation.

ϵ_{280}	nonlinear estimation	model comp. in 6M GdnHCl	model comp. in 8M Urea	exp. values
carbonic anhydrase (1)	49268 (962)	50066 (321)	49820 (322)	51540
CPA (1)	64630 (1377)	64362 (366)	64216 (368)	64999
CTgen (1)	50479 (1054)	51215 (357)	50836 (358)	50549
αlac (1)	28359 (603)	28371 (182)	28188 (183)	29075
βlgb (1)	17423 (434)	17011 (98)	16921 (98)	17474
lysozyme (4)	37921 (796)	38442 (268)	38156 (269)	36735
papain (1)	54189 (1258)	53251 (294)	53180 (296)	57582

RNase A (3)	9235 (441)	8227 (61)	8251 (62)	9228
BSA (4)	41255 (1622)	37952 (214)	37894 (216)	41372
HSA (1)	34413 (1571)	30984 (190)	30963 (191)	35245
RNase T_1 (10)	18330 (545)	17524 (102)	17549 (103)	17931
RNase Ba (1)	26022 (539)	26058 (151)	25988 (152)	25880
trypsin (1)	37099 (856)	36349 (204)	36208 (206)	36345
Therm (1)	54876 (1637)	53112 (315)	53252 (318)	52875
Staph Nuc (1)	15086 (417)	14698 (84)	14721 (85)	15610
BPTI (1)	6239 (309)	5526 (41)	5539 (41)	5435
insulin (1)	6239 (309)	5526 (41)	5539 (41)	5454

Abbreviations used: carboxypeptidase A (CPA); chymotrypsinogen (CTgen); α-lactalbumin (α-lac); β-lactoglobulin (β-lgb);

ribonuclease A (RNase A); bovine serum albumin (BSA); human serum albumin (HSA); ribonuclease T_1 (RNase T_1); ribonuclease Ba, also known as "barnase" (RNase Ba); thermolysin (Therm); staphylococcal nuclease (Staph Nuc); bovine pancreatic trypsin inhibitor (BPTI)

Table 5: Differences between experimental and estimated molar extinction coefficients for the 17 proteins in Table 4. Values given were calculated using the equation

$$\%\Delta\epsilon = \frac{\epsilon_{exp} - \epsilon_{est}}{\epsilon_{exp}} \times 100.$$

Average values were calculated by summing the absolute values of the differences and dividing by 17.

$\%\Delta\epsilon_{280}$	nonlinear estimation	6M GdnHCl	8M Urea
carbonic anhydrase	4.4	2.8	3.3
CPA	0.6	1.0	1.2
CTgen	0.1	-1.3	-0.6
αlac	2.5	2.4	3.0
βlgb	0.3	2.6	3.2
lysozyme	-3.2	-4.6	-3.9
papain	5.9	7.5	7.6
RNase A	-0.1	10.8	10.6
BSA	0.3	8.3	8.4
HSA	2.4	12.1	12.1
RNase T_1	-2.2	2.3	2.1

RNase Ba	-0.5	-0.7	-0.4
trypsin	-2.1	0.0	-0.4
Therm	-3.8	-0.4	-0.7
Staph Nuc	3.4	5.8	5.7
BPTI	-14.8	-1.7	-1.9
insulin	-14.4	-1.3	-1.6
avg. $\%\Delta\epsilon_{280}$	3.4	3.8	3.9

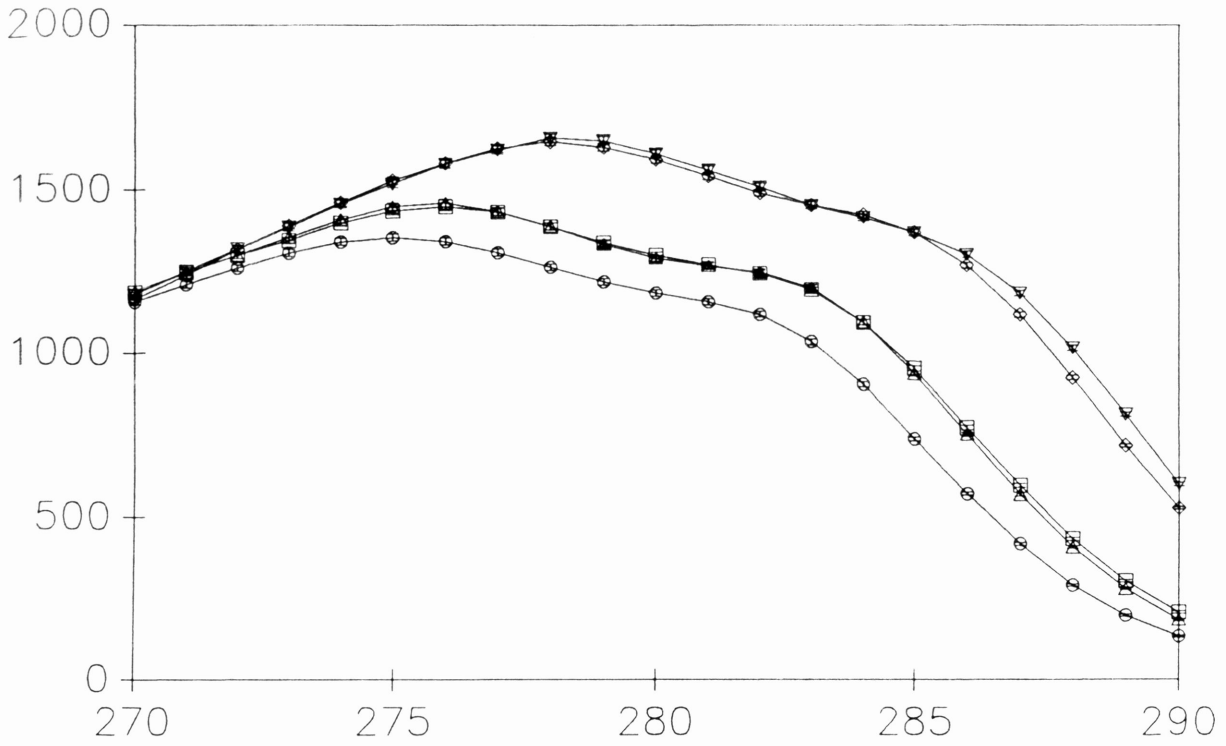

Figure 1: Absorption spectra for N-Ac-Tyr-OEE in water (circles), 6M GdnHCl (triangles), 8M urea (squares), 1-propanol (inverted triangles), and formamide (diamonds). Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

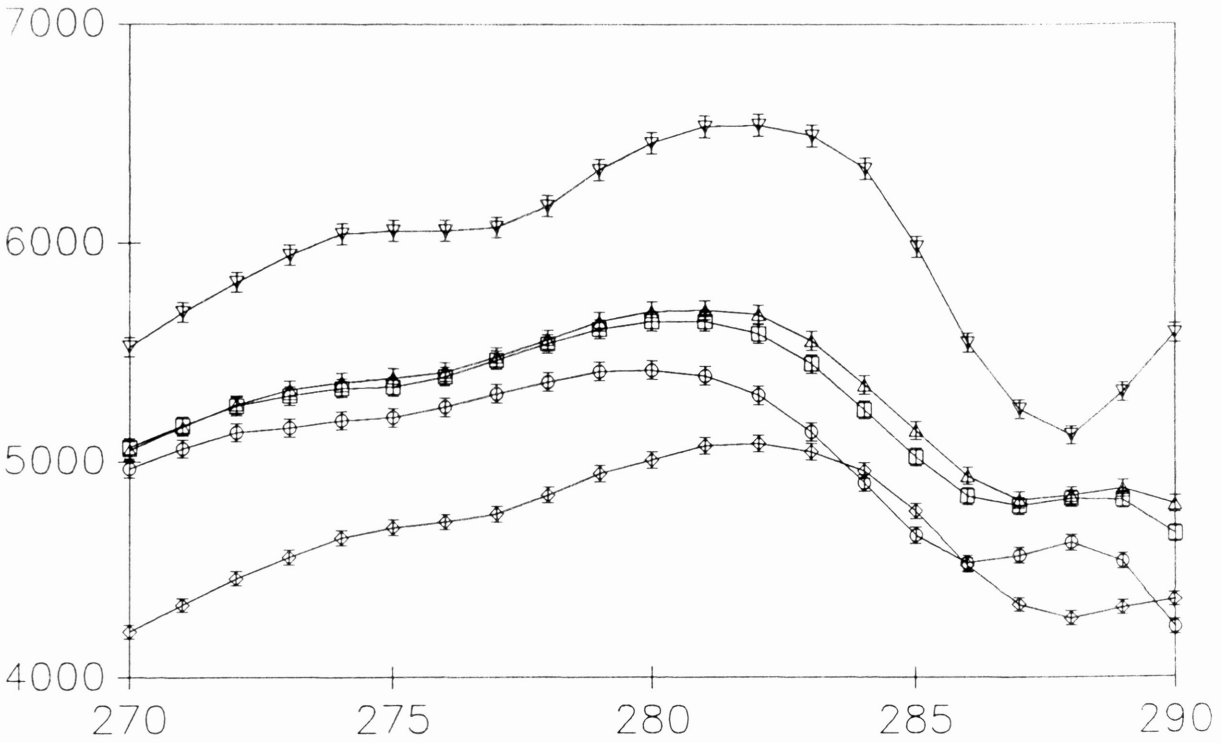

Figure 2: Absorption spectra for N-Ac-Trp-OEE in water (circles), 6M GdnHCl (triangles), 8M urea (squares), 1-propanol (inverted triangles), and formamide (diamonds). Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

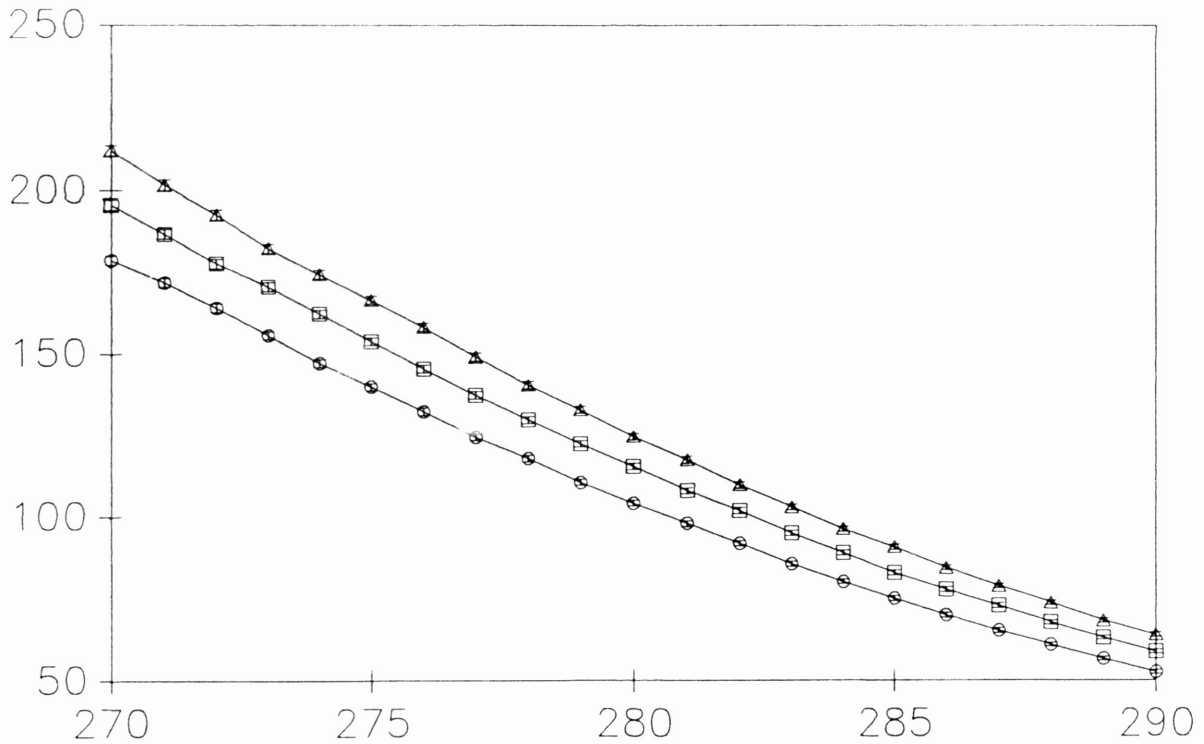

Figure 3: Absorption spectra for oxidized glutathione in water (circles), 6M GdnHCl (triangles), and 8M urea (squares). Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

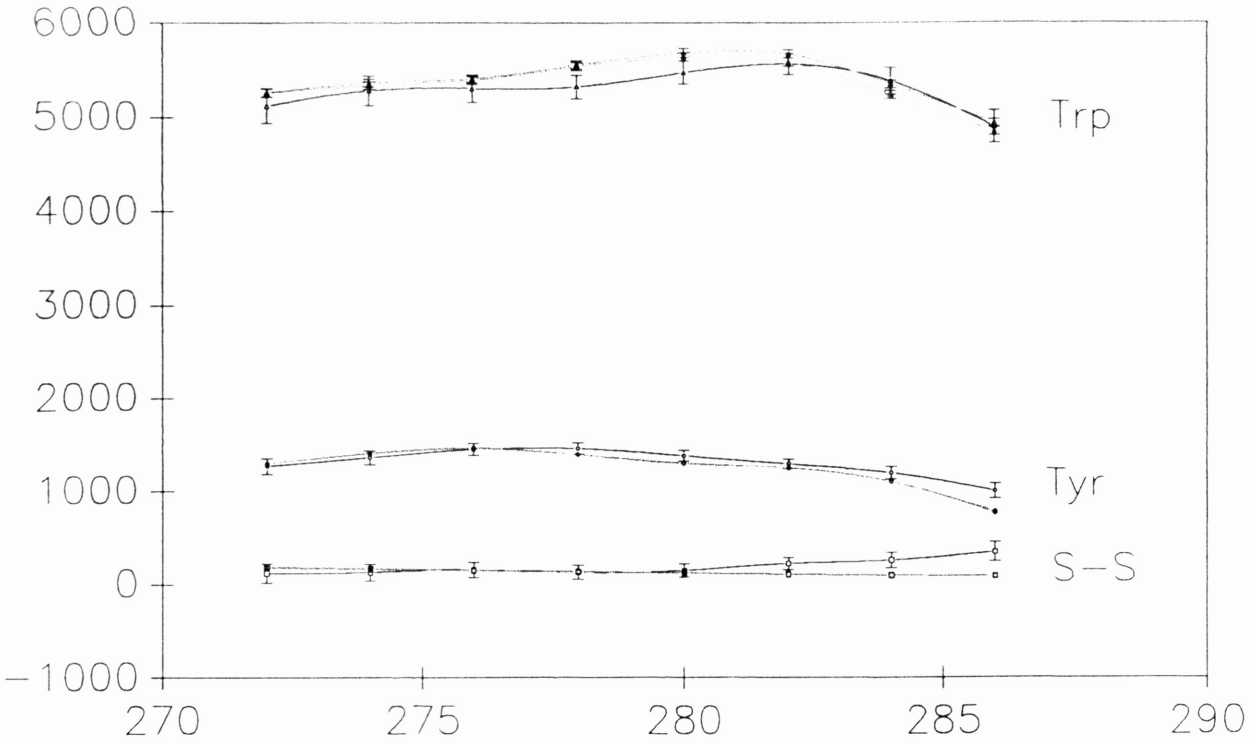

Figure 4: Absorption spectra for the chromophores Tyr, Trp, and disulfides (S-S). Dark lines represent the absorbance (molar) calculated using the NONLIN program. Dotted lines represent the absorbance measured using model compounds in 6 M GdnHCl and in 8 M urea. Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

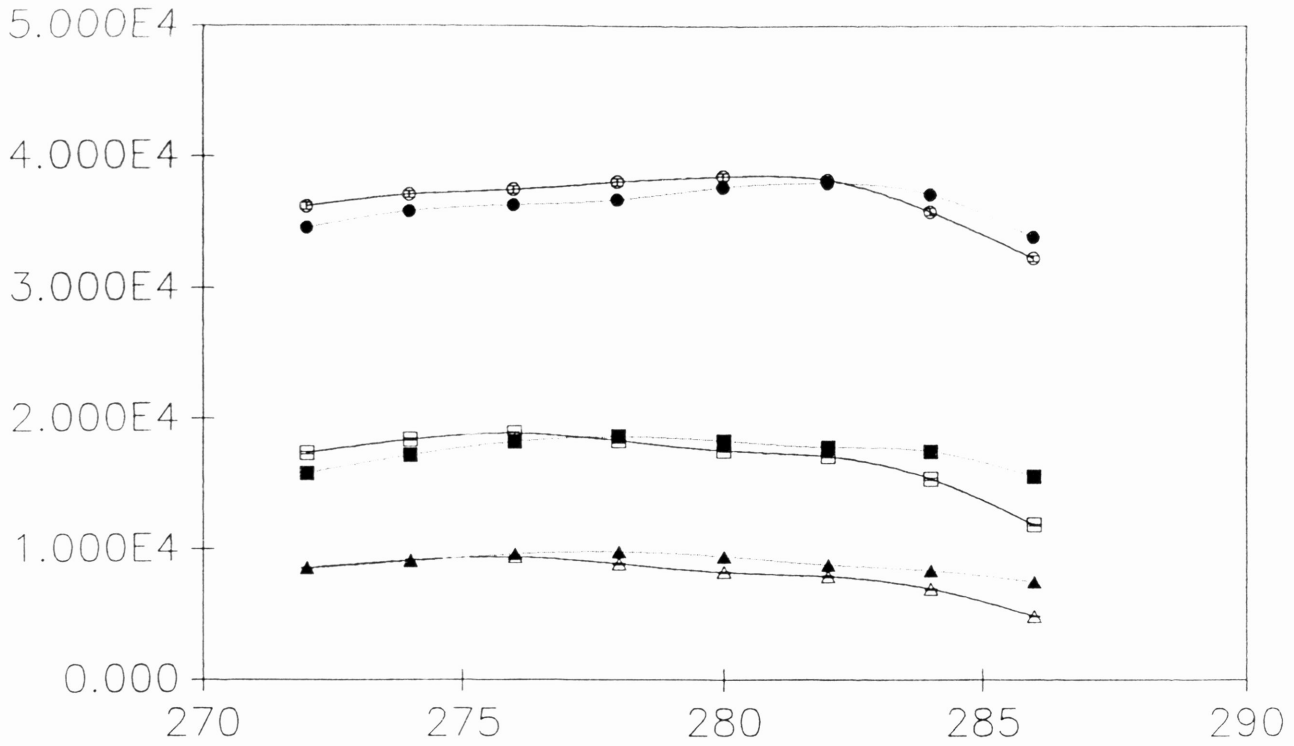

Figure 5: Absorption spectra for lysozyme (circles), RNase T_1 (squares), and RNase A (triangles). The clear symbols represent the estimated molar extinction coefficient based on model compounds in 6M gdnHCl, while filled symbols represent the experimental molar extinction coefficients at that wavelength. Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

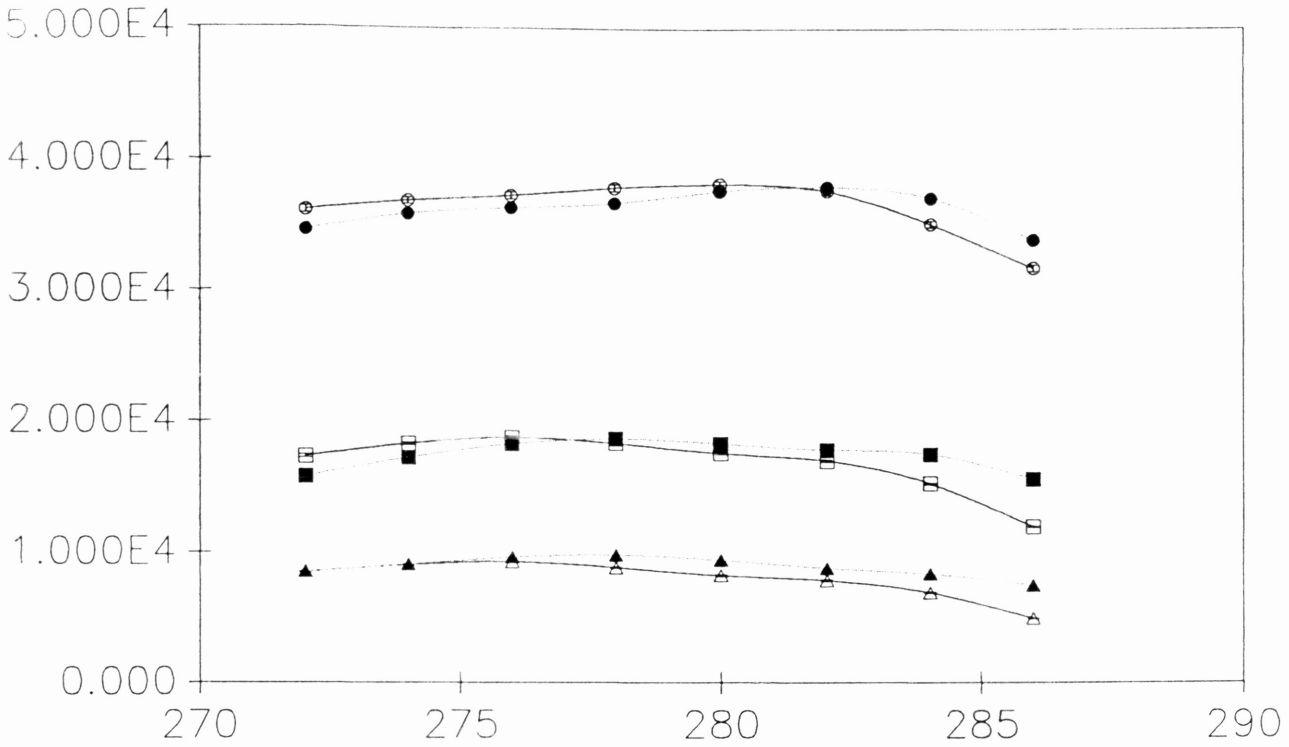

Figure 6: Absorption spectra for lysozyme (circles), RNase T_1 (squares), and RNase A (triangles). The clear symbols represent the estimated molar extinction coefficient based on model compounds in 8M urea, while filled symbols represent the experimental molar extinction coefficients at that wavelength. Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).

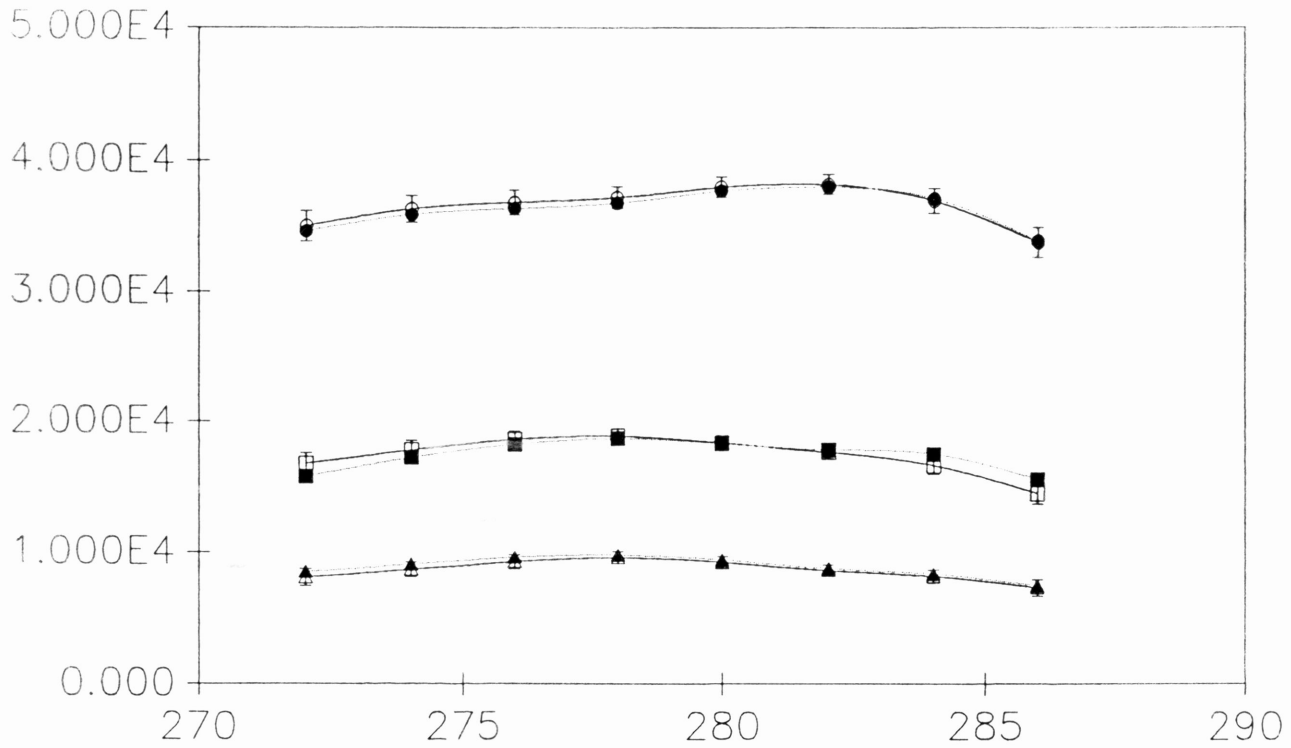

Figure 7: Absorption spectra for lysozyme (circles), RNase T_1 (squares), and RNase A (triangles). The clear symbols represent the estimated molar extinction coefficient based on nonlinear parameter estimation of equation (2), while filled symbols represent the experimental molar extinction coefficients at that wavelength. Horizontal axis is wavelength (in nm); vertical axis is the molar extinction coefficient (in $M^{-1}cm^{-1}$).