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How to measure and predict the molar absorption coefficient of a protein

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Abstract

The molar absorption coefficient, ϵ, of a protein is usually based on concentrations measured by dry weight, nitrogen, or amino acid analysis. The studies reported here suggest that the Edelhoch method is the best method for measuring ϵ for a protein. (This method is described by Gill and von Hippel [1989, *Anal Biochem 182*:319–326] and is based on data from Edelhoch [1967, *Biochemistry 6*:1948–1954].) The absorbance of a protein at 280 nm depends on the content of Trp, Tyr, and cystine (disulfide bonds). The average ϵ values for these chromophores in a sample of 18 well-characterized proteins have been estimated, and the ϵ values in water, propanol, 6 M guanidine hydrochloride (GdnHCl), and 8 M urea have been measured. For Trp, the average ϵ values for the proteins are less than the ϵ values measured in any of the solvents. For Tyr, the average ϵ values for the proteins are intermediate between those measured in 6 M GdnHCl and those measured in propanol. Based on a sample of 116 measured ϵ values for 80 proteins, the ϵ at 280 nm of a folded protein in water, $\epsilon(280)$, can best be predicted with this equation:

$$\epsilon(280)\ (M^{-1}\ cm^{-1}) = (\#Trp)(5{,}500) + (\#Tyr)(1{,}490) + (\#cystine)(125).$$

These $\epsilon(280)$ values are quite reliable for proteins containing Trp residues, and less reliable for proteins that do not. However, the Edelhoch method is convenient and accurate, and the best approach is to measure rather than predict ϵ.

Keywords: molar absorption coefficient; molar extinction coefficient; near UV absorbance; tryptophan absorbance; tyrosine absorbance

Biochemists must frequently determine the concentration of a protein solution. This is most often done by measuring the absorbance, A, near 280 nm and using the Beer-Lambert law:

$$A = \epsilon l C \qquad (1)$$

where ϵ is the molar absorption coefficient ($M^{-1}\ cm^{-1}$), l is the pathlength (cm), and C is the protein concentration (M). This is an excellent method for measuring protein concentrations provided that an accurate value of ϵ is available. The goal of the studies described here was to answer the following questions. First, what is the best experimental method for determining ϵ for a protein? Second, can ϵ be predicted accurately from the amino acid composition of a protein?

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To determine ϵ requires an accurate measurement of A and C (Equation 1). The measurement of A is straightforward (Schmid, 1989; Mach et al., 1995), but the measurement of C is not. The four techniques most often used to measure C are: amino acid analysis (Benson et al., 1975), Kjeldahl nitrogen determination (Jaenicke, 1974), the dry weight method (Hunter, 1966; Kupke & Dorrier, 1978; Nozaki, 1986), and the Edelhoch method (Edelhoch, 1967; Gill & von Hippel, 1989). Table 7 shows ϵ values determined for ribonuclease T1 over the years. It is clear that these techniques do not always lead to ϵ values in good agreement. The same is true for ribonuclease A (Wetlaufer, 1962) and other proteins (Gill & von Hippel, 1989). In this paper, we report ϵ values determined for RNase T1 and six mutants, and for seven other proteins by the dry weight and/or Edelhoch methods. We conclude that the Edelhoch method is the simplest and most reliable experimental method for determining ϵ.

The absorbance of a protein solution above 275 nm depends on just three chromophores: the side chains of Trp, Tyr, and

cystine (Wetlaufer, 1962). (Throughout this paper, cystine content is identical to disulfide bond content. In contrast to cystine residues, cysteine residues do not contribute significantly to the absorbance above 275 nm [Bailey, 1968].) The ϵ values for the protein chromophores depend on their environment (Yanari & Bovey, 1960; Brandts & Kaplan, 1973). Our main interest is in predicting the ϵ values for globular proteins. In globular proteins, the three contributing chromophores are most often buried in the interior of the protein: 87% buried for Trp, 76% buried for Tyr, and 92% buried for cystine (Lesser & Rose, 1990). Consequently, if we knew the solvent that best represented the interior of a folded protein, then we could measure ϵ values for the Trp, Tyr, and cystine chromophores in that solvent and might be able to use these values to predict ϵ values for proteins with acceptable accuracy. Wetlaufer (1962) and Perkins (1986) used ϵ values for model chromophores determined in water and showed that the ϵ values for proteins could be predicted reasonably well. More recently, Gill and von Hippel (1989) showed that ϵ values for the model chromophores determined in 6 M GdnHCl can also be used to predict ϵ values for proteins reasonably well. Most recently, Mach et al. (1992) used a statistical method to estimate the best average ϵ values at 280 nm for the three chromophores in a sample of 32 proteins and these further improved the fit between measured and predicted ϵ values for their sample of proteins. In this paper, we report ϵ values for Trp, Tyr, and cystine models measured in water, 8 M urea, 6 M GdnHCl, and propanol. In addition, we have analyzed the absorption spectra of a sample of 18 globular proteins to estimate the average ϵ values for Trp, Tyr, and cystine between 272 and 286 nm. Finally, we have analyzed a sample of 116 measured ϵ values for 80 different proteins to estimate the best ϵ values for Trp, Tyr, and cystine to predict ϵ values at 280 nm for proteins.

Results

Protein molar absorption coefficients determined by the dry weight and Edelhoch methods

Molar absorption coefficients for wild-type RNase T1 and six mutants determined in buffer and in 6 M GdnHCl by the dry weight method and in buffer by the Edelhoch method are listed in Table 1. For the RNase T1 values in the second row and for all of the mutants, the same absorbance measurements were used for both methods. The ϵ values in GdnHCl average 4.1% less than the values in buffer. In general, λ_{max} shifts to shorter wavelengths and ϵ decreases when proteins are dissolved in 6 M GdnHCl or 8 M urea (Sela et al., 1957; Lee & Timasheff, 1974; Prakash et al., 1981; Nozaki, 1986). For RNase T1 in buffer, the ϵ values from the Edelhoch method are always higher than ϵ values from the dry weight method. For wild-type RNase T1, the difference is about 5%, and only for Asp 49 Trp is the difference greater than 10%. Some of the factors that may contribute to the difference are considered in the Discussion. As a check on our dry weight method, ϵ values for RNase A and lysozyme were determined and the agreement with literature values was reasonably good (Table 2).

The results obtained over a period of years and summarized in Table 1 suggested that the Edelhoch method might be more reliable than the dry weight method for determining ϵ for a protein. To test this further, we measured ϵ values with the Edelhoch method for seven well-characterized proteins. The results are summarized in Table 2. The ϵ values based on the Edelhoch method are in excellent agreement with results from the literature determined by other methods. The average deviation is $\pm 2.0\%$. The largest deviations are for RNase A and β-lactoglob-

Table 1. *Molar absorption coefficients at 278 nm for RNase T1 and six mutants determined by the dry weight and Edelhoch methods*[a]

	Dry weight		Edelhoch
Protein	6 M GdnHCl	Buffer	Buffer
RNase T1	n.d.	18,520 ± 330	19,160 ± 270
RNase T1	17,420 ± 590	18,220 ± 390	19,300 ± 100
Asp 49 Ala	18,030	18,840	19,200
Asp 49 Phe	17,470 ± 590	18,310 ± 60	19,200 ± 1,130
Asp 49 Tyr	18,470 ± 490	19,300 ± 180	20,300 ± 50
Asp 49 Trp	21,700 ± 370	22,050 ± 210	24,500 ± 160
Trp 59 Tyr	13,620	14,080	14,700
Trp 59 Phe	12,000 ± 170	12,590 ± 190	13,800 ± 180
RNase T1 average[b]	17,640 ± 260	18,470 ± 210	19,215 ± 40

[a] Buffer = 30 mM MOPS, pH 7. 6 M GdnHCl = 6 M guanidine hydrochloride, 30 mM MOPS, pH 7. ϵ value = mean ± mean deviation. For the first entry, the dry weight result is based on three independent measurements and was reported in Hu et al. (1992), and the Edelhoch result is based on three independent measurements. For the other entries, the number of independent measurements was three for wild-type RNase T1, one for D49A, two for D49F, two for D49Y, three for D49W, one for W59Y, and two for W59F.

[b] ϵ value = mean ± mean deviation of the first four entries. D49A and D49F were included with wild type because they have the same content of Trp, Tyr, and cystine and they must be folded because they have greater enzyme activity than the wild-type enzyme.

Table 2. *Molar absorption coefficients for several proteins determined by the dry weight and Edelhoch methods*[a]

Protein	λ	Dry weight	Edelhoch	Literature
RNase T1	278	18,470 ± 210	19,215 ± 40	19,330 ± 1,040
Lysozyme	281	37,070 ± 450	38,010 ± 80	37,860 ± 450
RNase A	278	9,490 ± 160	9,460 ± 180	9,880 ± 150
BSA	278		44,600 ± 20	43,820 ± 530
β-lact	278		16,550 ± 80	17,460 ± 440
CTgen	282		50,380 ± 1,080	51,600 ± 880
BPTI	276		5,740 ± 10	5,700 ± 100
Insulin	276		6,020 ± 2	6,010 ± 230

[a] ϵ value = mean ± mean deviation. For RNase T1, the dry weight and Edelhoch values are from Table 1 and the literature value is from Table 7. For lysozyme, the ϵ values are based on three dry weight determinations, two Edelhoch determinations, and five literature values (2.65 ± 0.03 mL mg^{-1} cm^{-1}). For RNase A, the ϵ values are based on three dry weights, five Edelhoch determinations, and six literature values (0.722 ± 0.011 mL mg^{-1} cm^{-1}). For the other proteins, the ϵ values are based on two Edelhoch determinations, and the following ϵ values (mL mg^{-1} cm^{-1}) from the literature: BSA, 0.660 ± 0.008 for 10 ϵ values; β-lactoglobulin, 0.951 ± 0.024 for 5 ϵ values; chymotrypsinogen, 2.01 ± 0.04 for 3 ϵ values; BPTI, 0.867 ± 0.012 for 7 ϵ values; insulin, 1.04 ± 0.04 for 7 ϵ values.

ulin. For RNase A, $\epsilon = 9{,}800\ M^{-1}\ cm^{-1}$ at 277–278 nm is the ϵ value used most often over the years. Both our dry weight and Edelhoch values are about 4% less than this. Our dry weight results for RNase A yield $\epsilon = 9{,}870\ M^{-1}\ cm^{-1}$ if we do not correct the absorbances for light scattering. One possibility is that many of the literature values for RNase A were not corrected for light scattering and might be too high. For β-lactoglobulin, the ϵ value in common use is $17{,}550\ M^{-1}\ cm^{-1}$ at 278 nm (Townend et al., 1960). However, we determined a value of 16,650 $M^{-1}\ cm^{-1}$ if the absorbance values were corrected for light scattering (Pace, 1966; Cupo & Pace, 1983), in excellent agreement with our result from the Edelhoch method.

Model compound molar absorption coefficients

Absorption spectra for N-Ac-Trp-OEt and N-Ac-Tyr-OEt were measured in the following solvents: water, 8 M urea, 6 M GdnHCl, 1-propanol, dioxane, and formamide. Molar absorption coefficients calculated for the first four solvents at four wavelengths are given in Table 3. The results for dioxane and formamide were similar to the results for propanol. For Trp and Tyr, the values in 8 M urea and 6 M GdnHCl are essentially identical, and differ only slightly from the values in water. For Trp, λ_{max} is about 2 nm higher and ϵ_{max} about 9% greater in propanol than in water, and for Tyr, λ_{max} is about 3 nm higher and ϵ_{max} about 25% greater in propanol than in water. The ϵ values for oxidized glutathione in 6 M GdnHCl are similar to the values for cystine reported by Edelhoch (1967). Note that the ϵ values for oxidized glutathione are about 2% of the ϵ values for Trp, and about 9% of the ϵ values for Tyr. We also determined the absorption spectrum for N-Ac-Phe-OEt in water and found $\epsilon = 188\ M^{-1}\ cm^{-1}$ at $\lambda_{max} = 257.6$ nm, $\epsilon = 3.7\ M^{-1}\ cm^{-1}$ at 275 nm, and ϵ is less at longer wavelengths. Thus, the contribution of Phe to the absorption of proteins above 275 nm is negligible.

Average ϵ values between 272 and 286 nm for Trp, Tyr, and cystine based on the absorption spectra of 18 globular proteins

Absorption spectra from 350 to 250 nm were measured for 18 proteins: the first 11 in Table 5 plus trypsin, thermolysin, staphylococcal nuclease (SN), bovine pancreatic trypsin inhibitor (BPTI), insulin, RNase T1, and barnase. The protein concentration for each protein was based on the average of the most reliable ϵ values from the literature and is given in the Methods section. The ϵ values were calculated from 272 to 286 nm at 2-nm

Table 3. *Molar absorption coefficients of Trp, Tyr, and cystine model compounds in water, 8 M urea, 6 M GdnHCl, and propanol*[a]

Wavelength	Water	8 M urea	6 M GdnHCl	Propanol
		Tryptophan models[b]		
282	5,525	5,580	5,635	6,125
280	5,630	5,635	5,685	6,075
278	5,590	5,535	5,565	5,780
276	5,455	5,385	5,405	5,680
		Tyrosine models[c]		
282	1,145	1,240	1,220	1,560
280	1,215	1,300	1,285	1,680
278	1,295	1,390	1,395	1,735
276	1,390	1,450	1,455	1,660
		Cystine models[d]		
282	100	105	115	120
280	110	115	125	135
278	125	130	140	150
276	145	150	160	165

[a] The units for all values are $M^{-1}\ cm^{-1}$.

[b] The values given for water, 8 M urea, and propanol are the average of at least two measurements using N-Ac-Trp-OEt. The values given for 6 M GdnHCl are the average of our values and Edelhoch's values obtained with N-Ac-Trp-NH_2 and given in Gill and von Hippel (1989).

[c] The values given for water, 8 M urea, and propanol are the average of at least two measurements using N-Ac-Tyr-OEt. The values given for 6 M GdnHCl are the average of our values and Edelhoch's values for Gly-Tyr-Gly given in Gill and von Hippel (1989).

[d] The values given for cystine in water, 8 M urea, and 6 M GdnHCl are the average of at least two measurements using oxidized glutathione. The value given for cystine in propanol is the average of our values with cystine in propanol and Bailey's (1968) values with cystine in ethanol.

[e] Some literature values for ϵ_{max} and λ_{max} for comparison (see Nozaki, 1990, for other values):

N-Ac-Trp-OMe (water): 5,600 (279.8 nm) (Bailey, 1968)
N-Ac-Trp-NH_2 (6 M GdnHCl): 5,690 (280.8 nm) (Edelhoch, 1967)
N-Ac-Trp-OEt (propanol): 6,200 (282.5 nm) (Solli & Herskovits, 1973)
N-Ac-Tyr-OEt (water): 1,390 (274.6 nm) (Brandts & Kaplan, 1973)
N-Ac-Tyr-OMe (water): 1,420 (274.6 nm) (Bailey, 1968)
N-Ac-Tyr-OEt (6 M GdnHCl): 1,450 (275.3 nm) (Brandts & Kaplan, 1973)
N-Ac-Tyr-OEt (6 M GdnHCl): 1,500 (275.5 nm) (Edelhoch, 1967).
N-Ac-Tyr-OEt (propanol): 1,710 (277.8 nm) (Brandts & Kaplan, 1973)
N-Ac-Tyr-OEt (propanol): 1,710 (278.3 nm) (Solli & Herskovits, 1973)

Table 4. *Molar absorption coefficients for Trp, Tyr, and cystine based on an analysis of the absorption spectra of 18 proteins*[a]

Wavelength	Trp	Tyr	Cystine
	Cystine not fixed		
272	5,084 ± 170	1,405 ± 84	20 ± 108
274	5,248 ± 130	1,490 ± 63	65 ± 82
276	5,286 ± 107	1,563 ± 52	104 ± 67
278	5,315 ± 100	1,557 ± 48	170 ± 61
280	5,520 ± 106	1,457 ± 50	173 ± 65
282	5,603 ± 130	1,362 ± 63	182 ± 82
284	5,473 ± 201	1,234 ± 98	232 ± 126
286	5,026 ± 277	986 ± 135	354 ± 174
	Cystine fixed		
272	5,095 ± 238	1,345 ± 77	195
274	5,253 ± 176	1,452 ± 57	175
276	5,289 ± 134	1,545 ± 44	160
278	5,313 ± 120	1,568 ± 39	140
280	5,517 ± 128	1,474 ± 41	125
282	5,599 ± 164	1,387 ± 54	110
284	5,466 ± 262	1,281 ± 86	95
286	5,013 ± 346	1,078 ± 126	85

[a] These values in M^{-1} cm^{-1} are based on an analysis of the measured absorption spectra of the first 11 proteins in Table 5 plus those for trypsin, thermolysin, staphylococcal nuclease, BPTI, insulin, RNase T1, and barnase, as described in the text.

intervals, and the results were used to estimate the average ϵ values for Trp, Tyr, and cystine given in Table 4. The $\epsilon(\lambda)$ value for a protein can be calculated with

$$\epsilon(\lambda) = [\#\text{Trp}]\epsilon_\lambda(\text{Trp}) + [\#\text{Tyr}]\epsilon_\lambda(\text{Tyr}) + [\#\textit{cystine}]\epsilon_\lambda(\text{cystine}) \quad (2)$$

where #Trp, #Tyr, and #cystine are the numbers of residues of each type in the protein (see Table 5), and ϵ_λ(Trp, Tyr, or cystine) are the average ϵ values for these residues in the same protein. We have used the nonlinear least-squares method of Johnson and Frasier (1985) to find the values of ϵ_λ(Trp, Tyr, or cystine) that minimize the sum of the squares of the deviations of the $\epsilon(\lambda)$ values calculated with Equation 2 from the measured $\epsilon(\lambda)$ values. The results of this analysis are given in Table 4. In the top panel, the ϵ_λ(Trp, Tyr, and cystine) values were all allowed to vary. Note that the ϵ values for cystine increase with increasing wavelength. This is not reasonable, and probably results because cystine makes such a small contribution to $\epsilon(\lambda)$ that it cannot be estimated reliably. Consequently, we also did the analysis with ϵ_λ(cystine) fixed at the values determined for oxidized glutathione in 6 M GdnHCl (Table 3), and only the ϵ_λ(Trp) and ϵ_λ(Tyr) values were allowed to vary. These results are given in the lower panel of Table 4. We also showed that changing the ϵ value for a given protein by ±10% has only a small effect on the ϵ values for Trp and Tyr derived from the analysis. Note that

Table 5. *Observed and predicted molar absorption coefficients at 280 nm for 80 proteins*[a]

No.	Protein	MW	Trp	Tyr	Cystine	A(280,1%)	ϵ(obs)	ϵ(pred)	%Dev
1	CA (H-C)	29,118	7	8	0	17.70	51,539	50,420	2.17
2	CPA	34,409	7	19	1	18.90	65,033	66,935	−2.92
3	CTgen	25,666	8	4	5	19.70	50,562	50,585	−0.05
4	α-Lact	14,183	4	4	4	20.90	29,642	28,460	3.99
5	β-Lgb	18,300	2	4	2	9.63	17,623	17,210	2.34
6	Lysozyme	14,314	6	3	4	26.30	37,646	37,970	−0.86
7	Papain	23,426	5	19	3	24.60	57,628	56,185	2.50
8	RNase A	13,690	0	6	4	6.89	9,432	9,440	−0.08
9	BSA	66,400	2	20	17	6.61	43,890	42,925	2.20
10	HSA	66,450	1	18	17	5.30	35,219	34,445	2.20
11	3KI	13,400	0	3	0	3.36	4,502	4,470	0.72
12	Y14F	13,383	0	2	0	2.14	2,864	2,980	−4.05
13	Y55F	13,383	0	2	0	2.31	3,091	2,980	3.61
14	Y88F	13,383	0	2	0	2.48	3,319	2,980	10.21
15	Y55,88F	13,367	0	1	0	1.30	1,738	1,490	14.25
16	Y14,88F	13,367	0	1	0	1.23	1,644	1,490	9.38
17	Y14,55F	13,367	0	1	0	0.98	1,310	1,490	−13.74
18	Aldolase	39,000	3	12	0	9.10	35,490	34,380	3.13
19	Alc DHase(Y)	36,700	5	14	0	13.10	48,077	48,360	−0.59
20	CPA	34,414	7	19	1	17.90	61,601	66,935	−8.66
21	CPB	34,617	8	22	3	21.00	72,696	77,155	−6.13
22	CTgen	25,670	8	4	5	20.00	51,340	50,585	1.47
23	GAP DHase	35,606	3	11	0	8.90	31,689	32,890	−3.79
24	Glu DHase	55,755	4	18	0	9.20	51,295	48,820	4.82
25	Insulin	5,734	0	4	3	9.61	5,510	6,335	−14.96
26	Lac Rep	34,612	2	8	0	6.70	23,190	22,920	1.16
27	α-Lact	14,186	4	4	4	20.30	28,798	28,460	1.17
28	β-Lgb	18,285	2	4	2	9.60	17,554	17,210	1.96
29	Lysozyme	14,314	6	3	4	26.40	37,789	37,970	−0.48

(*continued*)

Table 5. *Continued*

No.	Protein	MW	Trp	Tyr	Cystine	$A(280,1\%)$	ϵ(obs)	ϵ(pred)	%Dev
30	T4 lysozyme	18,700	3	6	0	12.80	23,936	25,440	−6.28
31	Ovalbumin	45,900	3	10	1	7.01	32,176	31,525	2.02
32	Papain	23,426	5	19	3	24.80	58,096	56,185	3.29
33	RNase A	13,690	0	6	4	6.92	9,473	9,440	0.35
34	BSA	66,296	2	20	17	6.53	43,291	42,925	0.85
35	HSA	66,470	1	18	17	5.34	35,495	34,445	2.96
36	Tgen	23,998	4	10	6	15.10	36,237	37,650	−3.90
37	D-a.a. oxidase	39,336	10	14	0	18.60	73,165	75,860	−3.68
38	AAT	44,933	7	15	0	14.50	65,153	60,850	6.60
39	CA (H-B)	29,115	6	8	0	16.80	48,913	44,920	8.16
40	CA (B-B)	28,980	7	8	0	18.00	52,164	50,420	3.34
41	A-crystallin	19,790	1	6	0	8.00	15,832	14,440	8.79
42	B-crystallin	20,063	2	2	0	8.30	16,652	13,980	16.05
43	II-crystallin	21,003	4	15	0	21.00	44,106	44,350	−0.55
44	Myokinase	21,638	0	7	0	5.20	11,252	10,430	7.30
45	Thioredoxin	11,675	2	2	0	11.70	13,660	13,980	−2.34
46	Transferrin	75,181	8	26	5	11.10	83,451	83,365	0.10
47	Trypsin	23,993	4	10	6	15.70	37,669	37,650	0.05
48	Lysozyme	14,306	6	3	4	28.00	40,057	37,970	5.21
49	RNase A	13,690	0	6	4	7.02	9,610	9,440	1.77
50	α-CT	25,185	8	4	5	19.70	49,614	50,585	−1.96
51	Concan	25,572	4	7	0	13.70	35,034	32,430	7.43
52	SN	16,800	1	7	0	9.30	15,624	15,930	−1.96
53	Insulin	5,734	0	4	3	9.72	5,573	6,335	−13.66
54	Papain	23,426	5	19	3	24.60	57,628	56,185	2.50
55	Alc DHase(H)	39,794	2	4	0	4.55	18,106	16,960	6.33
56	Elastase	25,896	7	11	4	19.90	51,533	55,390	−7.48
57	PG Kinase	44,570	2	7	0	4.95	22,062	21,430	2.87
58	RNase T1	11,089	1	9	2	18.60	20,626	19,160	7.11
59	Barnase	12,383	3	7	0	22.10	27,366	26,930	1.59
60	ApoMb	17,201	2	3	0	9.00	15,481	15,470	0.07
61	Lysozyme	14,314	6	3	4	26.90	38,505	37,970	1.39
62	R-lysozyme	14,314	6	3	0	25.25	36,143	37,470	−3.67
63	RNase A	13,690	0	6	4	7.04	9,638	9,440	2.05
64	R-RNase A	13,690	0	6	0	6.98	9,556	8,940	6.44
65	SN	16,786	1	7	0	9.39	15,762	15,930	−1.07
66	ApoCyt *c*	11,702	1	4	0	9.20	10,766	11,460	−6.45
67	Barnase	12,383	3	7	0	20.90	25,880	26,930	−4.06
68	Barnase	12,383	3	7	0	22.10	27,366	26,930	1.59
69	W35F	12,344	2	7	0	17.50	21,602	21,430	0.80
70	W71F	12,360	2	8	0	18.90	23,360	22,920	1.89
71	W94F	12,344	2	7	0	18.20	22,466	21,430	4.61
72	W94L	12,310	2	7	0	18.10	22,281	21,430	3.82
73	RNase T1	11,089	1	9	2	17.50	19,406	19,160	1.27
74	RNase T1	11,089	1	9	2	17.30	19,184	19,160	0.12
75	D49A	11,045	1	9	2	17.40	19,218	19,160	0.30
76	D49F	11,121	1	9	2	17.40	19,351	19,160	0.98
77	D49Y	11,137	1	10	2	18.20	20,269	20,650	−1.88
78	D49W	11,160	2	9	2	22.00	24,552	24,660	−0.44
79	W59Y	11,066	0	10	2	13.30	14,718	15,150	−2.94
80	W59F	11,050	0	9	2	12.50	13,813	13,660	1.10
81	T4 lysozyme	18,636	3	6	0	13.00	24,227	25,440	−5.01
82	W138Y	18,613	2	7	0	11.30	21,033	21,430	−1.89
83	W126,138,158Y	18,567	0	9	0	6.84	12,700	13,410	−5.59
84	Lysozyme	14,314	6	3	4	26.20	37,503	37,970	−1.25
85	RNase A	13,690	0	6	4	7.03	9,624	9,440	1.91
86	BSA	66,400	2	20	17	6.47	42,961	42,925	0.08
87	β-Lgb	18,300	2	4	2	9.45	17,294	17,210	0.48
88	CTgen	25,666	8	4	5	19.80	50,819	50,585	0.46
89	BPTI	6,518	0	4	3	8.25	5,377	6,335	−17.81
90	Insulin	5,782	0	4	3	9.89	5,718	6,335	−10.78
91	Ovalbumin	45,900	3	10	1	6.91	31,717	31,525	0.61

(*continued*)

Table 5. *Continued*

No.	Protein	MW	Trp	Tyr	Cystine	A(280,1%)	ϵ(obs)	ϵ(pred)	%Dev
92	BSA	66,296	2	20	17	6.27	41,568	42,925	−3.27
93	CA (B-B)	28,980	7	8	0	16.90	48,976	50,420	−2.95
94	RNase A	13,690	0	6	4	6.68	9,145	9,440	−3.23
95	R-RNase A	13,690	0	6	0	6.25	8,556	8,940	−4.49
96	Helicase II	81,989	13	23	6	12.90	105,766	106,520	−0.71
97	Rep protein	76,400	9	22	0	10.05	76,782	82,280	−7.16
98	Luciferase A	40,108	6	16	0	13.40	53,745	56,840	−5.76
99	Luciferase A	40,108	6	16	0	14.10	56,552	56,840	−0.51
100	Luciferase B	36,349	2	10	0	7.30	26,535	25,900	2.39
101	Luciferase B	36,349	2	10	0	7.10	25,808	25,900	−0.36
102	Luciferase AB	76,457	8	26	0	11.50	87,926	82,740	5.90
103	Luciferase AB	76,457	8	26	0	11.40	87,161	82,740	5.07
104	DHFR (E.C.)	17,680	5	4	0	17.60	31,117	33,460	−7.53
105	DHFR (M)	21,446	3	6	0	11.70	25,092	25,440	−1.39
106	Thermolysin	34,334	3	28	0	16.60	56,994	58,220	−2.15
107	Peptidase A	25,165	5	14	3	18.30	46,052	48,735	−5.83
108	Neurotoxin	6,867	2	1	4	20.80	14,283	12,990	9.06
109	Glucagon	3,483	1	2	0	23.00	8,011	8,480	−5.86
110	Acid protease	34,239	4	14	2	12.60	43,141	43,110	0.07
111	Rhodanese	32,800	8	11	0	17.50	57,400	60,390	−5.21
112	Staphylokinase	15,660	1	9	0	11.70	18,322	18,910	−3.21
113	Glycerol kinase	56,106	13	18	0	17.50	98,186	98,320	−0.14
114	TPI (R)	26,750	5	4	0	12.90	34,508	33,460	3.04
115	β-Lgb	18,300	2	4	2	9.05	16,562	17,210	−3.92
116	PGK	44,570	2	7	0	4.95	22,062	21,430	2.87

[a] The name of the protein or an abbreviation is given in column 2. *Abbreviations:* CA(H-C), isozyme C of human carbonic anhydrase; CPA, bovine carboxypeptidase A; CTgen, bovine chymotrypsinogen; α-Lact, bovine α-lactalbumin; β-lgb, bovine β-lactoglobulin; BSA, bovine serum albumin; HSA, human serum albumin; 3-KI, Δ^5-3-ketosteroid isomerase; Alc DHase(Y), yeast alcohol dehydrogenase; CPB, bovine carboxypeptidase B; GAP DHase, yeast glyceraldehyde-3-phosphate DHase; Glu DHase, bovine glutamate dehydrogenase; Lac rep, *Escherichia coli* lac repressor; Tgen, bovine trypsinogen; AAT, chicken mitochondrial aspartate aminotransferase; CA(H-B), isozyme B of human carbonic anhydrase; A-crystallin, bovine α-A_2-crystallin; B-crystallin, bovine α-B_2-crystallin; II-crystallin, bovine γII-crystallin; α-CT, bovine α-chymotrypsin; Concan, jack bean concanavalin A; SN, staphylococcal nuclease; Alc DHase(H), horse liver alcohol dehydrogenase; PG kinase, yeast phosphoglycerate kinase; Mb, myoglobin; R-lysozyme, reduced lysozyme; R-RNase A, reduced RNase A; Cyt *c*, bovine cytochrome *c*; BPTI, bovine pancreatic trypsin inhibitor; CA(B-B) isozyme B of bovine carbonic anhydrase; DHFR (E.c.), *E. coli* dihydrofolate reductase; DHFR (M), muscle dihydrofolate reductase; TPI(R), rabbit triose phosphate isomerase; PGK, yeast phosphoglycerate kinase.

The molecular weight and the Trp, Tyr, and cystine contents are given in columns 3–6. We confirmed most of this information in at least one of the available databases, but there are still likely to be a few errors. In gathering ϵ values, we would generally start with the comprehensive compilation of Kirschenbaum (1978). We tried to use only ϵ values obtained by the dry weight method, amino acid analysis, Kjeldahl nitrogen determinations, or the Edelhoch method. In column 7, A(280,1%) is the absorbance of a 1% solution of the protein at 280 nm. Many of the A(280) values were determined at wavelengths other than 280 nm. To correct values at other wavelengths, $A(\lambda)$, to 280 nm, A(280), the following equation was used:

$$A(280) = [A(\lambda)]\ [\epsilon(280,\ 6\ \text{M GdnHCl})/\epsilon(\lambda,\ 6\ \text{M GdnHCl})],$$

where ϵ(280, 6 M GdnHCl) and $\epsilon(\lambda$, 6 M GdnHCl) are ϵ values for the protein at 280 nm and λ calculated with Equation 2 using the Trp, Tyr, and cystine content for each protein given in this table and the model compound data for GdnHCl in Table 3. In column 8, ϵ(obs) = [0.1][MW][A(280,1%)] is the molar absorption coefficient at 280 nm. In column 9, ϵ(pred) = 5,500(#Trp) + 1,490(#Tyr) + 125(#cystine) is the ϵ(pred) value for the protein based on the recommended ϵ values for Trp, Tyr, and cystine given in Table 9. In column 10, %Dev = 100[ϵ(obs) − ϵ(pred)]/ϵ(obs).

Some of the entries were taken from tables in other papers: 1–11 are from Table V in Wetlaufer (1962); 18–36 are from Table 3 in Gill and von Hippel (1989); and 37–47 are from Table 1 in Mach et al. (1992). In the latter two tables, we averaged the most reliable values when more than one ϵ value was given. Also, any errors we found in these tables were corrected. In Wetlaufer (1962), for example, the Trp, Tyr, and cystine content was correct for only 1 of the 11 entries, and in Gill and von Hippel (1989), all of the cystine contents were wrong.

Entries 11–17 are from Li et al. (1993), Kuliopulos et al. (1989), or from data supplied by Paul Talalay. For entry 12, Y14F denotes the mutant of 3-ketosteroid isomerase in which Tyr 14 is replaced by Phe. This same nomenclature was used throughout the table for other mutants. The ϵ values in entries 48–57 are from Kalnin et al. (1990). Entries 58–66 are from the Privalov lab (Privalov et al., 1989; Griko et al., 1994; Yu et al., 1994). For barnase and mutants, entry 67 is from Lees and Hartley (1966), and 68–72 are from Loewenthal et al. (1991). For RNase T1 and mutants, entries 73–80, the ϵ values are from Tables 1 and 7. The references for the rest of the entries are as follows: 81–83, Elwell and Schellman (1977); 84–90, Table 3 footnote; 91–93, Nozaki (1986); 94, 95, White (1961); 96, Runyon and Lohman (1989); 97, Amaratunga and Lohman (1993); 98–103, Sinclair (1995); 104, 105, C. Clark (pers. comm.); 106, Voordouw and Roche (1974); 107, Robinson (1975); 108, Hauert et al. (1974); 109, Kay and Marsh (1959); 110, Fukumoto et al. (1967); 111, P. Horowitz (pers. comm.); 112, Damaschun et al. (1993); 113, D. Pettigrew (pers. comm.), Pettigrew et al. (1988); 114, B. Gracy (pers. comm.); 115, Cupo and Pace (1983); 116, Adams et al. (1985).

the uncertainty in the ϵ value estimates is lowest near 278 nm, as expected, where it is ±2–3% for ϵ_λ(Trp) and ϵ_λ(Tyr).

Average ϵ values at 280 nm for Trp, Tyr, and cystine based on 116 measured ϵ values for 80 proteins

In order to estimate average ϵ values for Trp, Tyr, and cystine residues in globular proteins, we compiled the 116 measured ϵ values for 80 different proteins in Table 5. Similar but less extensive tables were compiled by Wetlaufer (1962), Gill and von Hippel (1989), and Mach et al. (1992). These tables all contained errors, and there are surely some errors in our table, but we doubt that they will significantly change the average ϵ values we derive from our analysis.

The analysis used above to estimate the ϵ values in Table 4 was applied to the data in Table 5 to obtain the results in Table 6. The top line shows the estimates obtained for ϵ(Trp), ϵ(Tyr), and ϵ(cystine) when all three are varied. Note that ϵ(cystine) = 128 M^{-1} cm^{-1} is in the range expected based on the results in Table 3. In rows 2 and 3, cystine is fixed at the extreme values suggested by Table 3 to show that the value assigned to ϵ(cystine) does not significantly effect the estimates of ϵ(Trp) and ϵ(Tyr) derived from the analysis. The fourth row gives the results of an analysis run using only the proteins that contain Trp residues. The ϵ value depends strongly on wavelength near 280 nm for proteins without Trp so that ϵ cannot be determined as accurately. Also, we will see that three of the four prominent outliers in our predicted values, 3-KI(Y14,55F), BPTI, and insulin, are proteins with no Trp residues. For this smaller sample of 93 ϵ values, the estimates of ϵ(Trp) and ϵ(Tyr) do not differ significantly from those for the complete data set. Thus, excluding proteins with Trp = 0 does not significantly change the results of the analysis. The fifth row gives the results obtained for the 23 proteins that contain no Trp. The ϵ estimate for Tyr differs by only about 2% from the estimate based on the entire data set. In rows 6 and 7, we subdivide the data set into approximately equal halves on the basis of cystine content. Here, the Trp estimates do not differ significantly, but the Tyr estimates differ by almost 4%. Finally, in the last row, we give an analysis of 11 proteins used by Wetlaufer (1962) to first test the possibility of estimating ϵ values for proteins using model compound data. We will see in Table 9 that these ϵ estimates for Trp, Tyr, and cystine are considerably better at predicting ϵ values for a protein than the ϵ values used by Wetlaufer (1962).

Table 6. *Estimated molar absorption coefficients for Trp, Tyr, and cystine at 280 nm based on analyses of the ϵ values in Table 5*

	Trp	Tyr	Cystine
116 ϵ values[a]	5,443 ± 141	1,503 ± 72	128 ± 104
Cystine fixed[b]	5,444 ± 157	1,501 ± 62	135
Cystine fixed[b]	5,437 ± 157	1,508 ± 62	110
Trp present[c]	5,434 ± 175	1,508 ± 71	125
Trp = 0[d]	–	1,472 ± 38	125
Cystine present[e]	5,430 ± 139	1,484 ± 52	125
Cystine = 0[f]	5,417 ± 257	1,540 ± 106	–
First 11 entries[g]	5,485 ± 129	1,507 ± 51	125

[a] These ϵ values at 280 nm in M^{-1} cm^{-1} were estimated by minimizing the sum of the square of deviations of the ϵ(280 nm) values calculated with Equation 2 from the measured ϵ(280 nm) values given in Table 5 using the nonlinear least-squares analysis of Johnson and Frasier (1985).

[b] The ϵ value for cystine was fixed at the extreme possible values (Table 3) to show that the ϵ values estimated for Trp and Tyr values are not significantly changed by the value chosen for ϵ for cystine.

[c] The 93 ϵ values for proteins containing Trp were included.

[d] The 23 ϵ values for proteins with Trp = 0 were included.

[e] The 56 ϵ values for proteins containing cystine were included.

[f] The 60 ϵ values for proteins with cystine = 0 were included.

[g] This analysis was run on the first 11 entries in Table 5. These 11 proteins were the proteins included in Table V in Wetlaufer's review (1962).

Discussion

Measuring the absorption coefficient of a protein

The ϵ value for RNase T1 has been measured by all of the standard methods over the years and the results are not in good agreement (Table 7). In the mid-1980s, we sent a sample of RNase T1 to Dr. Yas Nozaki, who had developed an improved procedure for determining dry weights on small protein samples (Nozaki, 1986). He found ϵ_{278} = 17,190 M^{-1} cm^{-1} for RNase T1 and this was reported in a paper describing the purification of recombinant RNase T1 (Shirley & Laurents, 1990). Because this differed by ≈20% from the value of 21,180 M^{-1} cm^{-1} (1.91 mL mg^{-1} cm^{-1}) that was in general use, ϵ was measured several times in our laboratory by the dry weight method and this resulted in the value of 18,520 M^{-1} cm^{-1} that was reported in the calorimetric studies of RNase T1 by Hu et al. (1992). In that paper, we found that $\Delta H_{van't\ Hoff} > \Delta H_{cal}$ and were not sure why. In trying to resolve this question, the Privalov laboratory measured ϵ = 20,630 M^{-1} cm^{-1} using amino acid analysis and a Kjeldahl nitrogen method (Yu et al., 1994). Their preferred method is the Kjeldahl procedure of Jaenicke (1974); we tried this method in our laboratory and found 19,630 M^{-1} cm^{-1}. For other reasons, we had begun a study of mutants of RNase T1 differing in their aromatic amino acid content. We replaced Trp 59, which is 100% buried, with Tyr and Phe, and we replaced the most hyperexposed residue in the protein,

Table 7. *Measured molar absorption coefficients at 278 nm for RNase T1*

Reference	ϵ (M^{-1} cm^{-1})	Method
Takahashi (1961)	18,520	?
Takahashi (1962)	21,180	?
Minato et al. (1966)	20,290	?
Shirley and Laurents (1990)	17,190	Dry weight
Okajima et al. (1990)	20,510	Amino acid analysis
Grunert et al. (1991)	17,300	Vacuum dried
Hu et al. (1992)	18,520 ± 330	Dry weight
	19,160 ± 270	Edelhoch method
Yu et al. (1994)	20,630 ± 780	Nitrogen analysis
	20,630 ± 1,110	Amino acid analysis
This paper	19,630 ± 670	Nitrogen analysis
	18,470 ± 210	Dry weight
	19,215 ± 40	Edelhoch method
Mean ± mean deviation	19,330 ± 1,040	

Asp 49, with Trp, Tyr, Phe, and Ala. The ϵ values for wild-type RNase T1 and these mutants were determined on the same stock solutions of protein using the same absorbance measurements by the dry weight and the Edelhoch methods. These results are summarized in Table 1. Based on this tortuous 10-year experience, we thought that the Edelhoch method might be the method of choice for determining the ϵ value for a protein. We will summarize some of our reasoning.

We have had only limited experience with amino acid analysis or Kjeldahl nitrogen determinations, but the accuracy of these methods does not seem adequate for determining an ϵ value for a protein. The average percent error for 16 amino acids determined by amino acid analyses at 62 different sites was 10.9% (1994 report from the Association of Biomolecular Resource Facilities on the quality of amino acid analyses). Similarly, with the Kjeldahl method, most often used with proteins, Jaenicke (1974) states: "The reproducibility in control analyses with added standards is ± 5%." This is the difference between the ϵ values determined by nitrogen analyses by our lab and the Privalov lab (Table 7).

We have had more experience with the dry weight method. Under optimal conditions, the precision in a given dry weight determination is about ±0.2% (Kupke & Dorrier, 1978), and we estimate the reproducibility of independent determinations to be ±2–3%. With RNase T1, we consistently observe lower ϵ values from the dry weight method than from the Edelhoch method (Table 1). The difference is not large; the values from the dry weight method average about 5% lower than the values from the Edelhoch method. We are not sure why, but there are more uncertainties with the dry weight method. Hunter's (1966) studies with the dry weight method led to these conclusions: (1) "The basic problem in dry weight determinations is a lack of adequate criteria for determining the completeness of water removal when this process may be attended by oxidative destruction of the protein;" and (2) " . . . the actual value obtained depended on the method of drying which had been employed. Different drying conditions gave dry weights which varied up to almost one part in a hundred. The best conditions for obtaining the true dry weight of a protein are somewhat uncertain and probably vary from protein to protein." We agree. Pace (1966) used dry weights to determine the concentration of β-lactoglobulin stock solutions over a period of years and concluded: " . . . the difficulty lies in the determination of protein concentrations by dry weights, where, although the precision was uniformly good, the accuracy was not." Here are some of the questions: First, does drying at 107 °C to a constant weight remove all of the water? Second, does extensive dialysis against distilled water or passing a protein through a mixed-bed ion exchange column remove all of the counterions? Third, why does the dry protein slowly begin to gain weight when drying is continued over a period of days? (It should be possible to answer some of these questions using mass spectroscopic techniques.) Because of these uncertainties and because the dry weight method is so time- and protein-consuming, it is clear that the Edelhoch method, if sufficiently accurate, would be the preferred method for measuring ϵ for a protein.

Edelhoch method

Edelhoch's 1967 paper describes a method for determining the Trp and Tyr content of proteins. The information in this paper was first used to determine ϵ values for proteins by groups at the University of Oregon (Butler et al., 1977; Elwell & Schellman, 1977), and has since become known as the Edelhoch method. A clear description of the method was given by Gill and von Hippel (1989). The basic assumption of the Edelhoch method is that ϵ values determined for model compounds for Trp, Tyr, and cystine in 6 M GdnHCl can be used to approximate the ϵ values for the same chromophores in a protein unfolded in 6 M GdnHCl. Figures 2–5 in Edelhoch's paper (1967) show that this is a reasonable assumption. They show that the spectrum of a protein in 6 M GdnHCl can be reproduced quite accurately using a mixture of model compounds for Trp, Tyr, and cystine in 6 M GdnHCl. The absorption spectrum of the model compound mixtures is shifted slightly to shorter wavelengths compared to the protein spectrum, but the magnitude of the absorbances near λ_{max} are very similar.

Further support for the basic assumption of the Edelhoch method comes from an analysis of 39 measured ϵ values on 27 different proteins in 6 M GdnHCl and 8 M urea. In addition to the 7 measured ϵ values in 6 M GdnHCl given in Table 1, we used 9 ϵ values measured in 6 M GdnHCl by Nozaki (1986), 9 ϵ values in 6 M GdnHCl from Lee and Timasheff (1974), 11 ϵ values in 8 M urea from Prakash et al. (1981), 2 ϵ values in 6 M GdnHCl from Span et al. (1974), and 1 ϵ value in 8 M urea from Sela et al. (1957). We fixed $\epsilon = 125\ M^{-1}\ cm^{-1}$ at 280 nm for cystine (Table 3) for reasons explained above. We found that $\epsilon_{280} = 5{,}450\ M^{-1}\ cm^{-1}$ for Trp, and $\epsilon_{280} = 1{,}265\ M^{-1}\ cm^{-1}$ for Tyr minimized the percent deviation between the ϵ values calculated with Equation 2 and the 39 ϵ values measured in 6 M GdnHCl and 8 M urea. This Trp value is about 4% lower and the Tyr value about 2% lower than the values given in Table 3 for 6 M GdnHCl. This is reasonable agreement considering the uncertainty in the experimental ϵ values for proteins in 6 M GdnHCl and 8 M urea, and shows that the ϵ values for Trp, Tyr, and cystine in proteins unfolded in these solvent are similar to the ϵ values for model chromophores in the same solvents.

Our results on RNase T1 with the Edelhoch method (Table 1) encouraged us to try the method on some other well-characterized proteins. These results are summarized in Table 2. These values were obtained by preparing a solution of the protein, filtering the solution, diluting the solution into buffer and 6 M GdnHCl solutions, and measuring the absorption spectra of these two solutions. All of this can be done in a few hours. For these eight proteins, the average percent difference between results from the Edelhoch method and results from the literature is 2.0%. The only deviation greater than 5% is for β-lactoglobulin, and, as explained in the Results, we think this is because the literature ϵ value is too high. Thus, we conclude that the Edelhoch method is both the simplest and most accurate method for determining the molar absorption coefficient for a protein.

Predicting the absorption coefficient of a protein

We first measured the ϵ values for the model compounds for Trp, Tyr, and cystine (Table 3) in order to estimate the 278/251 absorbance ratio expected for pure RNase T1. We used this ratio to monitor our success in removing a yellow pigment during the purification of RNase T1 (Pace et al., 1987). At the time, we thought it might be possible to use the ϵ values in water to model the exposed chromophores in a protein, and the ϵ values in propanol to model the buried chromophores in a protein and

that this might allow us to calculate ϵ values for proteins with reasonable accuracy. As illustrated in Table 8, the results were not encouraging. Propanol was a surprisingly poor model for buried Trp and Tyr chromophores. Consequently, we needed a better approach for estimating the average ϵ values for the Trp, Tyr, and cystine chromophores in globular proteins, and this led us to the experiments summarized in Table 4.

The ϵ values in Table 4 are the average values for Trp, Tyr, and cystine that can best reproduce the measured absorption spectra for 18 well-characterized proteins using Equation 2. In Figure 1, we compare the absorption spectra based on the spectral properties of Trp and Tyr in these 18 proteins to the absorption spectra for Trp and Tyr in propanol and 6 M GdnHCl. For Tyr, the average spectral properties in the proteins are intermediate between the spectral properties in propanol and 6 M GdnHCl. For Trp, the spectral properties in proteins are closer to those observed in 6 M GdnHCl than those observed in propanol. In numerical terms, the ϵ values obtained from an analysis of the proteins (Tables 4, 6) are more than 10% lower than the ϵ values measured in propanol (Table 3). The ϵ value for Trp in proteins is particularly surprising because it is less than the value measured in any of the solvent systems, including water. It is not clear to us why these largely buried Trp residues behave more like they are in water than in a nonpolar environment. Richards (1977) has pointed out that "The interior of a protein is not an oil drop but resembles rather a molecular crystal," and this might explain why propanol is such a poor model for the interior of a protein, at least with regard to the spectral properties of Trp and Tyr. These results explain why ϵ values based on 6 M GdnHCl are reasonably successful in predicting ϵ values for folded proteins (Gill & von Hippel, 1989). Note in Table 8 that the ϵ values for Trp and Tyr based on proteins are much better at predicting the ϵ value for RNase T1 than those based on any of the solvent systems.

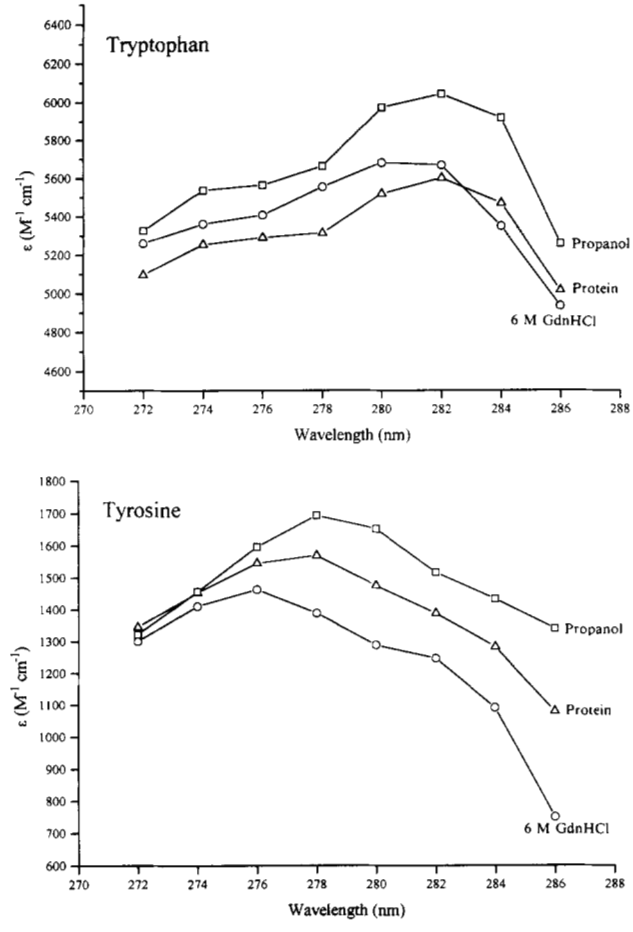

Fig. 1. Absorption spectra for tryptophan and tyrosine. The curves labeled 6 M GdnHCl and propanol are based on the absorption spectra used to determine the results given in Table 3. The curve labeled protein is based on the results in Table 4.

Table 8. *Estimating the molar absorption coefficient of RNase T1 at 278 nm*[a]

	Buried (propanol)	Exposed (water)
Trp (1)	1.0 × 5,780 = 5,780	0.0 × 5,590 = 0
Tyr (9)	7.7 × 1,735 = 13,360	1.3 × 1,295 = 1,684
S-S (2)	1.6 × 150 = 240	0.4 × 125 = 50
Total	19,380 +	1,734 = 21,114

Model	ϵ(calculated)
Water (Table 3)	17,495 (9.1% low)
6 M GdnHCl (Table 3)	18,380 (4.5% low)
Propanol (Table 3)	21,695 (12.7% high)
Propanol (buried) + water (exposed)	21,114 (9.7% high)
Protein (Table 4)	19,200 (≈equal)
ϵ(measured) (Table 1)	19,215 M^{-1} cm^{-1}

[a] All of the ϵ values are in M^{-1} cm^{-1}. The upper portion of the table shows the calculation of ϵ at 278 nm for RNase T1 using the ϵ values for water for the exposed chromophores and the ϵ values for propanol for the buried chromophores. The percent buried was calculated using the Lee and Richards program (Richards, 1977) and the 9RST crystal structure of RNase T1 from the Brookhaven Protein Data Bank (Martinez-Oyanedel et al., 1991). The lower portion shows the calculated absorption coefficients using ϵ values from the tables noted. The last line shows the measured ϵ value.

These results encouraged us to examine a larger sample of proteins. We compiled a list of 116 measured ϵ values for 80 different proteins (Table 5). We focused our attention on 280 nm, where most ϵ values are measured. When an ϵ value was measured at a wavelength other than 280 nm, it was corrected to 280 nm using the content of Trp, Tyr, and cystine and the ϵ values in 6 M GdnHCl given in Table 3, as explained in Table 5. The error introduced by this correction will generally be less than 2%. In Table 6, we show the values of ϵ for Trp, Tyr, and cystine that minimize the sum of the squares of the deviations of ϵ values calculated with Equation 2 from the measured values given in Table 5. The first row is based on an analysis of all 116 ϵ values in Table 5. The ϵ values are determined to ±2.6% for Trp, to ±4.8% for Tyr, and to ±81% for cystine. The second and third rows show that the ϵ value assigned to Cys does not significantly change the ϵ values estimated for Trp and Tyr. Consequently, we fixed $\epsilon = 125$ for cystine for the rest of the analyses. The next five rows analyze various subsets of the 116 ϵ values ranging in size from 11 to 93 entries. The estimates of ϵ for Trp range from 5,417 to 5,485 (1.3% difference), and the estimates for Tyr range from 1,472 to 1,540 (4.6% difference) for the var-

ious subsets and the complete data set. Thus, we doubt that the ϵ values estimated for Trp, Tyr, and cystine would change appreciably if a larger set of ϵ values were analyzed.

The average percent deviation, ave%dev, is a different measure of how well a given set of ϵ values for Trp, Tyr, and cystine will give agreement between ϵ(obs) and ϵ(pred) (Table 9). It should be a better measure because each ϵ value will be weighted more equally than in the least-squares method of Johnson and Frasier (1985) that was used to obtain the results in Tables 4 and 6. In Table 9, we show the ave%dev obtained using measured and estimated ϵ values for Trp, Tyr, and cystine from various sources. The first four lines show the ave%dev obtained using the ϵ values for Trp, Tyr, and cystine measured in four solvents (Table 3). The fifth line shows the result for the ϵ values used by Wetlaufer to predict ϵ values in his 1962 review. It is interesting that 8 M urea and 6 M GdnHCl are better model solvents for the buried chromophores of a protein than water or propanol. However, as the rest of Table 9 shows, none of the solvent systems allow us to predict ϵ values for folded proteins nearly as well as the ϵ estimates for Trp, Tyr, and cystine derived from analyzing proteins directly.

The ϵ values from Table 6 that were based on our analysis of all of the proteins in Table 5 gave an ave%dev = 3.856%. Note, however, that the ave%dev is slightly smaller using ϵ values derived from an analysis of just the first 11 proteins in Table 5. This shows that the combination of ϵ values that gives the best fit using the least-squares program of Johnson and Frasier (1985) does not give the minimum value for the ave%dev. Note also that the ϵ values that Mach et al. (1992) derived from a similar analysis of a list of 81 measured ϵ values for 32 different proteins gave ave%dev = 3.860%. This shows that quite different sets of ϵ values for Trp, Tyr, and cystine can give comparable values for ave%dev.

We next used trial and error to search for rounded values of ϵ for Trp and Tyr that would give an ave%dev less than those obtained using the ϵ values from Tables 4 and 6. The best combination that we found was ϵ_{Trp} = 5,500 M^{-1} cm^{-1}, ϵ_{Tyr} = 1,490 M^{-1} cm^{-1}, and $\epsilon_{cystine}$ = 125 M^{-1} cm^{-1} (ave%dev = 3.836), and this is denoted as recommended at the bottom of Table 9. In the last column in Table 5, we list the percent deviation, %Dev, of the measured ϵ values, ϵ(obs), from the ϵ values calculated with Equation 2, ϵ(pred), when these values are used. (Another possible combination was 5,450 for Trp, 1,500 for Tyr, and 125 for cystine [ave%dev = 3.850].) This is encouraging. It shows that we can predict ϵ reasonably well knowing just the Trp, Tyr, and cystine content of the protein. As we will now see, we can do even better if the protein contains at least one Trp.

Table 9. *Average percent deviation of measured ϵ values in Table 5 from predicted ϵ values using various Trp, Tyr, and cystine ϵ values*[a]

Source	Trp	Tyr	Cystine	Ave%dev[b]
6 M GdnHCl (Table 3)	5,685	1,285	125	6.881
8 M urea (Table 3)	5,635	1,300	115	6.700
Water (Table 3)	5,630	1,215	110	9.188
Propanol (Table 3)	6,075	1,680	135	11.858
Wetlaufer (1962)	5,550	1,340	150	5.894
Table 6	5,443	1,503	128	3.856
Table 6 (first 11 entries)	5,485	1,507	125	3.852
Table 4	5,517	1,474	125	3.900
Mach et al. (1992)	5,540	1,480	134	3.860
Recommended	5,500	1,490	125	3.836

[a] The ϵ values are in M^{-1} cm^{-1}. Predicted values were calculated with Equation 2 using the ϵ values given here and the number of Trp, Tyr, and cystine residues for each protein in Table 5.

[b] Ave%dev = $(1/116)\Sigma$ |%Dev|. %Dev = $100[\epsilon(obs) - \epsilon(pred)]/\epsilon(obs)$.

The only Trp-containing protein that gave a percent deviation greater than 10% was α-B_2-crystallin (entry 42 in Table 5). This ϵ value came from the list of Mach et al. (1992), and they point out that the light-scattering correction for this protein was 7%, greater than for any other protein in their list. Thus, the ϵ value for this protein is surely less reliable than most. In contrast, 7 of 23 entries with no Trp residues had percent deviations greater than 10%, with BPTI the greatest at −17.81% (Table 5). Using our recommended values for Trp, Tyr, and cystine, the ave%dev = 3.167% for the 93 entries containing Trp, but 6.541% for the 23 entries with Trp = 0. Thus, it is clear that we can predict ϵ values for proteins containing Trp much better than we can predict ϵ values for proteins lacking Trp. This is not surprising.

Based on our recommended ϵ values, a tryptophan residue is equivalent to 3.7 tyrosine residues and 44 cystine residues. Furthermore, it is clear from the model compound data that the ϵ values for Trp are less sensitive to the environment than the ϵ values for Tyr (Table 3). This is even clearer when the protein data are considered. For the three 3-KI mutants containing a single Tyr residue and no Trp or cystine residues (entries 15–17 in Table 5), the ϵ(280) values are 1,310, 1,640, and 1,740 M^{-1} cm^{-1}. For the 14 proteins in Table 5 with no Trp residues, the average ϵ(280) value per Tyr = 1,490 ± 140 M^{-1} cm^{-1}. (A correction for cystine was made using ϵ(280) = 125 M^{-1} cm^{-1} [Table 3].) Thus, the average Tyr in RNase A has ϵ(280) = 1,490 M^{-1} cm^{-1}, and the ϵ(pred) values for RNase A are in good agreement with the ϵ(obs) values; but for the average Tyr in BPTI, ϵ(280) = 1,270, and for the average Tyr in insulin, ϵ(280) = 1,290, and their %Dev values in Table 5 are the major outliers. Thus, in folded proteins there is a wider range of ϵ values for Tyr residues than there is for Trp residues and this is the main reason that it is more difficult to predict ϵ values for proteins that contain no Trp residues. (See Brandts and Kaplan [1973] for an excellent discussion of the spectral properties of Tyr residues in RNase A, insulin, and BPTI.)

In summary, we suggest using this equation:

$$\epsilon(280\text{ nm}) = 5{,}500(\#\text{Trp}) + 1{,}490(\#\text{Tyr}) + 125(\#\text{cystine})$$

to predict the ϵ value for a folded protein in water at 280 nm. It is quite reliable for proteins that contain Trp residues, and less reliable for proteins that do not. However, the Edelhoch method is convenient and accurate, and the best approach is still to measure rather than predict ϵ. If you are unlucky and have a protein with no Trp or Tyr, the paper by Scopes (1974) should be consulted.

Materials and methods

The water used was purified by reverse osmosis and then double-distilled in a glass still. MOPS buffer was purchased from Sigma. Urea was purchased from United States Biochemicals

(ultrapure), and from Sigma (ultra). Guanidine hydrochloride was purchased from Heico (extreme purity), and from United States Biochemicals (ultrapure). 1-Propanol was purchased from Fisher. Most of the model compound spectra were determined with these compounds: *N*-acetyl-tyrosine ethyl ester (N-Ac-Tyr-OEt) from American Tokyo Kasei; *N*-acetyl-tryptophan ethyl ester (N-Ac-Trp-OEt), oxidized glutathione (Grade III and ultrapure), and cystine (ultrapure) from Sigma. The proteins used were from the following sources: ribonuclease T1 and mutants prepared as described by Shirley and Laurents (1990); barnase prepared as described by R.W. Hartley (pers. comm.); β-lactoglobulin A prepared as described by Aschaffenburg and Drewry (1957); β-lactalbumin prepared as described by Robbins and Kronman (1964); ribonuclease A (Sigma, R-5500 and Type XIIA); hen lysozyme (Worthington, 2× crystallized); human carbonic anhydrase II (Sigma, C-6165); carboxypeptidase A (Sigma, 6510); chymotrypsinogen (Worthington, 5× crystallized, and Sigma Type II); papain (Sigma, P-4762); human serum albumin (Sigma, A-3782); bovine serum albumin (Calbiochem, 12659, and Sigma, A-4503); trypsin (Sigma, T-8253); thermolysin (Sigma, T-1512); staphylococcal nuclease (a kind gift from Dr. David Shortle); bovine pancreatic trypsin inhibitor (Sigma, T-0256, and Boehinger Mannheim); ovalbumin (Worthington, 2× crystallized); and bovine insulin (Sigma, I-5500).

Model compound spectra

The model compounds were dried in a vacuum desiccator over phosphorus pentoxide. Stock solutions of N-Ac-Trp-OEt, N-Ac-Tyr-OEt, oxidized glutathione, and cystine were prepared by weight. The UV spectra (250–350 nm) were measured at room temperature with a Cary model 15 or model 219 spectrophotometer.

Dry weight method

The dry weight procedure used to determine protein concentrations was based on a method used routinely in the Charles Tanford laboratory that came earlier from the Cohn and Edsall laboratory (Robinson, 1975; Nozaki, 1986). Kupke and Dorrier (1978) called a method similar to this "the commonly taught method of yesteryear," and stated that it has a precision of about 1 part in 90. (Detailed descriptions of the dry weight method can be found in Hunter [1966], Kupke and Dorrier [1978], and Nozaki [1986].) The dry weight method used to determine the ϵ values given in Tables 1 and 2 is as follows. Protein was dissolved in water to a concentration of ≈5 mg/mL and filtered through a 0.2-μm Acrodisc filter. This solution was then dialyzed against at least three changes of water to become our protein stock solution. Aliquots of the stock solution were weighed into carefully cleaned, dried, and tared weighing bottles. The solutions were first evaporated to dryness at ≈100 °C, and then dried further in a vacuum oven at 20 torr and 107 °C. The samples usually reach a constant weight within about 12 h, but heating was continued for 1–2 days to be sure that a constant weight was attained. Under these conditions, the increase in weight that is observed at longer times is not significant during 2 days of drying. Typically, the dry weights were run on triplicate samples, each containing enough protein solution that at least 10 mg of protein were present after drying. The weighing bottles were cooled in a greaseless desiccator before weighing on a Mettler balance accurate to ±0.01 mg. The stock solution used for determining the dry weights was diluted into 30 mM MOPS buffer, pH 7, and into the same buffer containing 6 M GdnHCl to give solutions with absorbances between 0.5 and 0.9, and their spectra were recorded from 250 to 350 nm with a Cary 15 spectrophotometer. When the solutions showed significant light scattering, i.e., significant absorption in the 320–350-nm region, a correction was applied as described by Leach and Scheraga (1960). This information was then used with Equation 1 to calculate the ϵ values determined by the dry weight procedure given in Tables 1 and 2.

Edelhoch method

The results for the Edelhoch procedure given in rows 2–8 in Table 1 were determined on the same stock solutions used for the dry weight procedure described above. For the other results from the Edelhoch procedure in Tables 1 and 2, the stock solution was prepared by simply filtering the protein solution through a 0.2-μm acrodisc filter. Identical dilutions of the protein stock solution were made into 30 mM MOPS buffer at pH 7 (buffer), and into the same buffer containing 6 M GdnHCl (6MG). The absorption spectra of the protein in buffer and 6MG was measured between 250 and 350 nm. If these solutions showed absorbance above ≈325 nm, then the absorbance measurements near 280 nm were corrected for a contribution from light scattering as described above. (The simplest method to correct for light scattering is to multiply the absorbance at 330 nm by 1.929 to get the light-scattering contribution at 280 nm or by 1.986 to get the light-scattering contribution at 278 nm. Alternatively, 2 times the absorbance at 333 nm gives the scattering contribution at 280 nm, and 2 times the absorbance at 331 nm gives the scattering contribution at 278 nm. These approaches assume that the scattering contribution varies as the inverse fourth power of the wavelength as in Rayleigh scattering.) At the wavelength where the absorbance is maximal in 6MG, ϵ_λ(6MG) is calculated using:

$$\epsilon_\lambda(6MG) = (\#Trp)\epsilon_\lambda(Trp,6MG) + (\#Tyr)\epsilon_\lambda(Tyr,6MG) + (\#cystine)\epsilon_\lambda(cystine,6MG),$$

where the ϵ_λ values in 6 M GdnHCl for Trp, Tyr, and cystine were taken from Table 3. Now the protein concentration in the 6 M GdnHCl solution can be calculated using:

$$C(6MG) = A(6MG)/\epsilon(6MG),$$

and this gives the protein concentration in buffer, C(buffer) = C(6MG). The ϵ value at any wavelength for the folded protein in buffer, ϵ_λ(buffer), can then be calculated using:

$$\epsilon_\lambda(buffer) = A_\lambda(buffer)/C(buffer).$$

Absorption spectra of various proteins

The results in Table 4 are based on an analysis of the absorption spectra of the following proteins (the ϵ value in M^{-1} cm^{-1} used to calculate the protein concentration is given in parentheses): carbonic anhydrase (human C) (51,540 at 280 nm); carboxypeptidase A (66,750 at 278 nm); chymotrypsinogen (51,330

at 282 nm); α-lactalbumin (29,070 at 280 nm); β-lactoglobulin (17,630 at 278 nm); lysozyme (37,640 at 280 nm); papain (58,570 at 278 nm); RNase A (9,800 at 278 nm); bovine serum albumin (44,070 at 278 nm); human serum albumin (36,040 at 278 nm); 3-ketosteroid isomerase (4,935 at 278 nm) (the absorption spectrum for this protein was kindly provided by Dr. Paul Talalay); trypsin (36,480 at 278 nm); thermolysin (58,650 at 278 nm); staphylococcal nuclease (16,220 at 278 nm); bovine pancreatic trypsin inhibitor (5,700 at 276 nm); insulin (6,070 at 276 nm); RNase T1 (19,290 at 278 nm); and barnase (27,370 at 280 nm). The absorption spectra between 250 and 350 nm for each protein was recorded using a Cary 15 spectrophotometer. The absorbance values at each wavelength were corrected for light scattering as described above. The protein concentration was calculated using the ϵ values and wavelengths given above, and then the ϵ values at 2-nm intervals between 272 nm and 286 nm were calculated using Equation 1.

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References

Adams B, Burgess RJ, Pain RH. 1985. The folding and mutual interaction of the domains of yeast phosphoglycerate kinase. *Eur J Biochem 152*: 715–720.

Amaratunga M, Lohman TM. 1993. *Escherichia coli* Rep helicase unwinds DNA by an active mechanism. *Biochemistry 32*:6815–6820.

Aschaffenburg R, Drewry J. 1957. Improved method for the preparation of crystalline β-lactoglobulin and α-lactalbumin from cow's milk. *Biochem J 65*:273–277.

Bailey JE. 1968. UV absorption characteristics of N-acetyl methyl esters of the aromatic amino acids, cystine, and N-acetylcysteine. In: Sober HA, ed. *Handbook of biochemistry*. Cleveland, Ohio: Chemical Rubber Co. p B-18.

Benson AM, Suruda AJ, Talalay P. 1975. Concentration-dependent association of Δ-3-ketosteroid isomerase of *Pseudomonas testosteroni*. *J Biol Chem 250*:276–280.

Brandts JF, Kaplan LJ. 1973. Derivative spectroscopy applied to tyrosyl chromophores. Studies on ribonuclease, lima bean inhibitors, insulin, and pancreatic trypsin inhibitor. *Biochemistry 12*:2011–2024.

Butler AP, Revzin A, von Hippel PH. 1977. Molecular parameters characterizing the interaction of *Escherichia coli* lac repressor with non-operator DNA and inducer. *Biochemistry 16*:4757–4768.

Cupo JF, Pace CN. 1983. Conformational stability of mixed disulfide derivatives of β-lactoglobulin B. *Biochemistry 22*:2654–2658.

Damaschun G, Damaschun H, Gast K, Misselwitz R, Zirwer D, Guhrs KH, Hartmann M, Schlott B, Triebel H, Behnke D. 1993. Physical and conformational properties of staphylokinase in solution. *Biochim Biophys Acta 1161*:244–248.

Edelhoch H. 1967. Spectroscopic determination of tryptophan and tyrosine in proteins. *Biochemistry 6*:1948–1954.

Elwell ML, Schellman JA. 1977. Stability of phage T4 lysozymes. Native properties and thermal stability of wild type and two mutant lysozymes. *Biochim Biophys Acta 494*:367–383.

Fukumoto J, Tsuru D, Yamamoto T. 1967. Studies on mold protease. Part I. Purification, crystallization, and some enzymatic properties of acid protease of *Rhizopus chinensis*. *Agr Biol Chem 31*:710–717.

Gill SC, von Hippel PH. 1989. Calculation of protein extinction coefficients from amino acid sequence data. *Anal Biochem 182*:319–326.

Griko YV, Makhatadze GI, Privalov PL, Hartley RW. 1994. Thermodynamics of barnase unfolding. *Protein Sci 3*:669–676.

Grunert HP, Zouni A, Beineke M, Quaas R, Georgalis Y, Saenger W, Hahn U. 1991. Studies of RNase T1 mutants affecting enzyme catalysis. *Eur J Biochem 197*:203–207.

Hauert J, Maire M, Sussmann A, Bargetzd JP. 1974. The major lethal neurotoxin of the venom of *Naja naja philippinensis*. *J Pept Protein Res 6*:201–222.

Hu CQ, Sturtevant JM, Thomson JA, Erickson RE, Pace CN. 1992. Thermodynamics of ribonuclease T1 denaturation. *Biochemistry 31*: 487–4882.

Hunter MJ. 1966. A method for the determination of protein partial specific volumes. *J Phys Chem 70*:3285–3292.

Jaenicke L. 1974. A rapid micromethod for the determination of nitrogen and phosphate in biological material. *Anal Biochem 61*:623–627.

Johnson ML, Frasier SG. 1985. Nonlinear least-squares analysis. *Methods Enzymol 117*:301–342.

Kalnin NN, Baikalov IA, Venyaminov SY. 1990. Quantitative IR spectrophotometry of peptide compounds in water solutions. III. Estimation of protein secondary structure. *Biopolymers 30*:1273–1280.

Kay CM, Marsh MM. 1959. Some optical properties of fetuin and glucagon. *Biochim Biophys Acta 33*:251–253.

Kirschenbaum DM. 1978. Molar absorptivity and A(1 cm, 1%) values for proteins at selected wavelengths of the ultraviolet and visible regions. *Anal Biochem 90*:309–330.

Kuliopulos A, Mildvan AS, Shortle D, Talalay P. 1989. Kinetic and ultraviolet spectroscopic studies of active-site mutants of Δ-3-ketosteroid isomerase. *Biochemistry 28*:149–159.

Kupke DW, Dorrier TE. 1978. Protein concentration measurements: The dry weight. *Methods Enzymol 48*:155–162.

Leach SJ, Scheraga HA. 1960. Effect of light scattering on ultraviolet difference spectra. *J Am Chem Soc 82*:4790–4792.

Lee JC, Timasheff SN. 1974. Partial specific volumes and interactions with solvent components of proteins in guanidine hydrochloride. *Biochemistry 13*:257–265.

Lees CW, Hartley RW. 1966. Studies on *Bacillus subtilis* ribonuclease. III. Purification and amino acid composition. *Biochemistry 5*:3951–3960.

Lesser GJ, Rose GD. 1990. Hydrophobicity of amino acid subgroups in proteins. *Proteins Struct Funct Genet 8*:6–13.

Li YK, Kuliopulos A, Mildvan AS, Talalay P. 1993. Environments and mechanistic roles of the tyrosine residues of Δ-3-ketosteroid isomerase. *Biochemistry 32*:1816–1824.

Loewenthal R, Sancho J, Fersht AR. 1991. Fluorescence spectrum of barnase: Contributions of three tryptophan residues and a histidine-related pH dependence. *Biochemistry 30*:6775–6779.

Mach H, Middaugh CR, Lewis RV. 1992. Statistical determination of the average values of the extinction coefficients of tryptophan and tyrosine in native proteins. *Anal Biochem 200*:74–80.

Mach H, Volkin DB, Burke CJ, Middaugh CR. 1995. Ultraviolet absorption spectroscopy. *Methods Mol Biol 40*:91–113.

Martinez-Oyanedel J, Choe HW, Heinemann U, Saenger W. 1991. Ribonuclease T1 with free recognition and catalytic site: Crystal structure analysis at 1.5 Å resolution. *J Mol Biol 222*:335–352.

Minato S, Tagawa T, Nakanishi K. 1966. Crystallization of ribonuclease T1. *J Biochem 59*:443–448.

Nozaki Y. 1986. Determination of the concentration of protein by dry weight—A comparison with spectrophotometric methods. *Arch Biochem Biophys 249*:437–446.

Okajima T, Kawata Y, Hamaguchi K. 1990. Chemical modification of tryptophan residues and stability changes in proteins. *Biochemistry 29*:9169–9175.

Pace CN. 1966. The reversible denaturation of β-lactoglobulin A [dissertation]. Durham, North Carolina: Duke University.

Pace CN, Grimsley GR, Barnett BJ. 1987. Purification of ribonuclease T1. *Anal Biochem 167*:418–422.

Perkins SJ. 1986. Protein volumes and hydration effects. The calculations of partial specific volumes, neutron scattering matchpoints and 280-nm absorption coefficients for proteins and glycoproteins from amino acid sequences. *Eur J Biochem 157*:169–180.

Pettigrew DW, Ma DP, Conrad CA, Johnson JR. 1988. *Escherichia coli* glycerol kinase: Cloning and sequencing of the *glpK* gene and the primary structure of the enzyme. *J Biol Chem 263*:135–139.

Prakash V, Loucheux C, Scheufele S, Gorbunoff MJ, Timasheff SN. 1981. Interactions of proteins with solvent components in 8M urea. *Arch Biochem Biophys 210*:455–464.

Privalov PL, Tiktopulo EI, Venyaminov SY, Griko YV, Makhatadze GI, Khechinashvili NN. 1989. Heat capacity and conformation of proteins in the denatured state. *J Mol Biol 205*:737–750.

Richards FM. 1977. Areas, volumes, packing, and protein structure. *Annu Rev Biophys Bioeng 6*:151–176.

Robbins FM, Kronman MJ. 1964. A simplified method for preparing α-lactalbumin and β-lactoglobulin from cow's milk. *Biochem Biophys Acta 82*: 186.

Robinson GW. 1975. Isolation and characterization of papaya peptidase A from commercial chymopapain. *Biochemistry 14*:3695–3700.

Runyon GT, Lohman TM. 1989. *Escherichia coli* helicase II (UvrD) protein can completely unwind fully duplex linear and nicked circular DNA. *J Biol Chem 264*:17502–17512.

Schmid FX. 1989. Spectral methods of characterizing protein conformation and conformational changes. In: Creighton TE, ed. *Protein structure: A practical approach*. Oxford, UK: IRL Press. pp 251–285.

Scopes RK. 1974. Measurement of protein by spectrophotometry at 205 nm. *Anal Biochem 59*:277–282.

Sela M, Anfinsen CB, Harrington WF. 1957. The correlation of ribonuclease activity with specific aspects of tertiary structure. *Biochim Biophys Acta 26*:502–512.

Shirley BA, Laurents DV. 1990. Purification of recombinant ribonuclease T1 expressed in *Escherichia coli*. *J Biochem Biophys Methods 20*:181–188.

Sinclair JF. 1995. Equilibrium and kinetic studies of the folding of the subunits of bacterial luciferase [dissertation]. College Station, Texas: Texas A&M University.

Solli NJ, Herskovits TT. 1973. Solvent perturbation studies and analysis of protein and model compound data in denaturing organic solvents. *Anal Biochem 54*:370–378.

Span J, Lenarcic S, Lapanje S. 1974. Solvation of lysozyme and β-lactoglobulin in aqueous guanidine hydrochloride solutions. *Biochim Biophys Acta 359*:311–319.

Takahashi K. 1961. The structure and function of ribonuclease T1. I. Chromatographic purification and properties of ribonuclease T1. *J Biochem 49*:1–8.

Takahashi K. 1962. The structure and function of ribonuclease T1. II. Further purification and amino acid composition of ribonuclease T1. *J Biochem 51*:95–107.

Townend R, Winterbottom RJ, Timasheff SN. 1960. Molecular interactions in β-lactoglobulin. II. Ultracentrifugation and electrophoretic studies of the association of β-lactoglobulin below its isoelectric point. *J Am Chem Soc 82*:3161–3168.

Voordouw G, Roche RS. 1974. The cooperative binding of two calcium ions to the double site of apothermolysin. *Biochemistry 13*:5017–5021.

Wetlaufer DB. 1962. Ultraviolet spectra of proteins and amino acids. *Adv Protein Chem 17*:303–391.

White FH. 1961. Regeneration of native secondary and tertiary structures by air oxidation of reduced ribonuclease. *J Biol Chem 236*:1353.

Yanari S, Bovey FA. 1960. Interpretation of the ultraviolet spectral changes of proteins. *J Biol Chem 235*:2818–2826.

Yu Y, Makhatadze GI, Pace CN, Privalov PL. 1994. Energetics of ribonuclease T1 structure. *Biochemistry 33*:3312–3319.