Spectrophotometric Titrations of Human Serum Albumin and Reduced Carboxymethylated Albumin

Abstract. *The tyrosyl groups of reduced and carboxymethylated human serum albumin, in which all disulfide bonds are broken, ionize at lower* pH *than those of native albumin, in spite of the greater negative charge on the protein produced by the S-carboxymethyl groups. The heat and entropy of ionization of these groups in the reduced albumin are also "normal," in contrast to native albumin. Spectrophotometric titrations at 244 and 295 mμ give equivalent results. The more open structure of the reduced albumin, as compared with the native protein, presumably allows the tyrosine groups to come freely into contact with solvent. In acid solutions the heights of the two peaks at 236 and 287 mμ in the difference spectrum (albumin at* pH *7 against albumin at a lower* pH*) are strictly proportional over the whole course of the titration. Both peaks apparently arise from changes in the environment of tyrosyl residues, rather than from more general conformational changes.*

Serum albumin consists of a single long peptide chain internally cross-linked by many disulfide bonds (*1*). The phenolic groups of the tyrosyl residues in bovine albumin, although they ionize reversibly, are characterized by an unusually high intrinsic *pK* value and by abnormally large heats and small negative entropies of ionization (*2*). If the constraints due to the disulfide bonds are broken, how are these properties of the tyrosyl groups affected? We have studied this question by comparing normal human serum albumin (HSA) with reduced carboxymethylated albumin (RCA), in which all disulfide bonds are broken, each being replaced by two S-carboxymethylcysteine residues.

Our studies also give at least partial answers to two questions concerning the ultraviolet spectra of serum albumins: (i) Do the absorption bands at 244 and 295 mμ increase in parallel as the phenolic groups ionize? (ii) Do the peaks at 236 and 287 mμ in the acid difference spectra (*3*) appear to have a common origin?

We dialyzed human serum albumin, a reworked preparation of fraction V of human plasma (*4*), extensively and deionized it on a mixed bed ion-exchange resin (*5*). We prepared reduced carboxymethylated albumin from it by a modification of the method of Jirgensons and Ikenaka (*6*) involving reduction with mercaptoethanol in 8*M* urea and treatment with iodoacetate at *p*H 8.5. The protein was isolated by precipitation at −5°C with acetone containing 0.05*N* HCl, and then it was washed with this solvent and cold ether. Amino acid analysis on a Beckman Spinco amino acid analyzer gave a value of 37.8 ± 1.7 moles of carboxymethylcysteine per 69,000 g of protein (*7*). The close agreement of this figure with the generally accepted figure for the half-cystine plus cysteine content of HSA (*1*) indicates that carboxymethylation was complete. Thus the reduced form contains approximately 37 more carboxyl groups than HSA, and at *p*H > 7 these should all bear negative charges.

We used a Cary recording spectrophotometer, model 11, for most measurements, and a Zeiss PMQ spectrophotometer for the difference spectra involving urea solutions. Observations of difference spectra at different protein concentrations showed that optical artifacts were absent.

Reduced carboxymethylated albumin, unlike the corresponding derivative prepared by reaction of reduced serum albumin with iodoacetamide (*8*), is soluble in aqueous solutions at *p*H above 6.5 and below 1.8, up to concentrations of at least 0.13 g/100 ml, the concentration used in many of our difference spectra. It is insoluble at intermediate *p*H values. For HSA we found $E^{1\%}_{1\,cm} = 5.3$ at 279 mμ, a value in complete agreement with that of Cohn *et al.* (*9*). The value of λ_{max} for RCA is 277.0 ± 0.3 mμ, 2.0 mμ below λ_{max} for HSA; White has observed a similar shift on reduction of ribonuclease (*10*).

For control of *p*H we employed as buffers glycine (*p*H 2 to 4 and 8.5 to 10.5), acetate (*p*H 4 to 6), phosphate (*p*H 6 to 8), ε-aminocaproate (*p*H 10 to 12) with total buffer concentration (acid plus conjugate base) generally 0.05*M*. We adjusted all solutions to ionic strength 0.1 with KCl, except the 0.15*N* NaOH solutions.

Human serum albumin contains 18 tyrosine residues, and the value for RCA is presumably the same. We observed the ionization of the phenolic groups in alkaline solution by measuring the change in molar absorptivity ($\Delta\varepsilon$) with *p*H of the large peak at 244 mμ (*11*) and the smaller peak at 295 mμ in the difference spectrum. The ratio $\Delta\varepsilon_{244}/\Delta\varepsilon_{295}$ remained constant at 4.6 ± 0.15 over the whole range of titration. Hermans (*11*) reports this ratio as 4.5 for sperm whale myoglobin and 4.2 for ribonuclease. Like Tanford and Roberts (*2*) we found that absorption in the more alkaline solutions, especially above *p*H 12, increased rapidly with time; the values extrapolated to zero time approach a constant level ($\Delta\varepsilon_{max}$) at high *p*H. This is represented by the values found in 0.15*N* NaOH recorded in Table 1; they are the same within experimental error for HSA and RCA. From these maxima we calculate $\alpha = \Delta\varepsilon/\Delta\varepsilon_{max}$ as the fraction of the phenolic groups ionized at

Table 1. Values of various difference spectra.

λ (mμ)	Sample solution	Reference solution	Δε
244	HSA, 0.15*N* NaOH	HSA, *p*H 7.0	178,000* ± 8000
295	HSA, 0.15*N* NaOH	HSA, *p*H 7.0	39,500* ± 2000
244	RCA, 0.15*N* NaOH	RCA, *p*H 7.0	175,000* ± 8000
244	BMA†, 0.15*N* NaOH	BMA, *p*H 7.0	225,000* ± 9000
246	Tyrosine, 0.15*N* NaOH	Tyrosine, *p*H 6.0	11,000 ± 300
236	HSA, *p*H 1.60	HSA, *p*H 7.0	−12,200 ± 1000
236	HSA, *p*H 1.2 + 8*M* urea	HSA, *p*H 1.2	− 8,900 ± 1000
236	RCA, *p*H 1.23	RCA, *p*H 7.0	−11,500 ± 1000
236	RCA, *p*H 1.23 + 8*M* urea	RCA, *p*H 1.23	−12,000 ± 2500

* Values extrapolated to zero time. † BMA; bovine serum mercaptalbumin.

Table 2. Ionization of phenolic groups in RCA measured at two temperatures and two wavelengths. ΔH° of ionization (see text) is calculated from the temperature coefficient of *p*H at constant α (see Tanford and Roberts, *2*). The standard entropy of ionization is given by $\Delta S° = (\Delta H° - 2.3\,RT\,pK_o)/T$. If the intrinsic *pK* (pK_o) is taken as 10.0, ΔS° = −31 e.u. If $pK_o = 9.6$, ΔS° = −29 e.u.

Degree of ionization	*p*H at 6.7°C		*p*H at 25.0°C	
	$\Delta\varepsilon_{244}$	$\Delta\varepsilon_{296.5}$	$\Delta\varepsilon_{244}$	$\Delta\varepsilon_{296.5}$
0.25	10.37	10.40	10.17	10.19
0.50	10.76	10.76	10.57	10.57

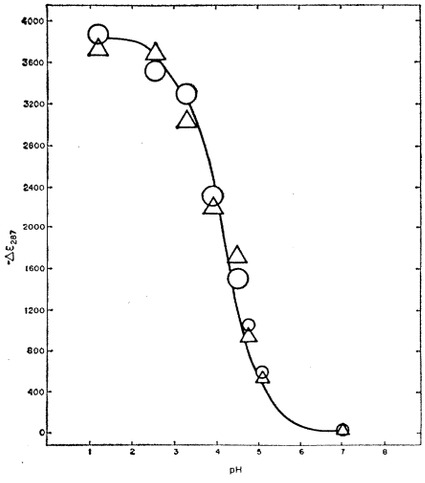

Fig. 1. Difference spectrum of human serum albumin in acid solution at 287 and 236 mμ. Circles denote values of $-\Delta\varepsilon_{287}$, that is, $-(\varepsilon_{287}$ at indicated *p*H $- \varepsilon_{287}$ at *p*H 7). Triangles denote corresponding values of $-\Delta\varepsilon_{236}$, divided by 3.28.

other *p*H values. In two different titrations at 25°C we found the *p*H at the midpoint ($\alpha = 0.5$) for HSA to be 11.69 and 11.90, respectively. Tanford and Roberts (*2*) found a slightly lower value (11.50) for bovine serum albumin at ionic strength 0.15.

The behavior of RCA (Table 2) is strikingly different; the *p*H value for $\alpha = 0.5$ is 10.57 ± 0.07, more than one *p*H unit lower than for HSA. This shift is in the opposite direction to what might be expected on purely electrostatic grounds; the added negative charges in RCA would tend to make ionization of the phenolic groups more difficult. The observed effect must be due to the fact that the constraints imposed by the disulfide bonds in the native molecule (HSA) have been released in RCA. Whether these constraints are due to hydrogen bonding, or to shielding of the tyrosyl groups in HSA by surrounding hydrophobic groups, or to a combination of the two effects, it is clear that the far more open structure of RCA renders the phenolic groups more accessible to the solvent, so that they ionize more readily. The increase in negative charge in alkaline solution due to the added S-carboxymethyl groups would promote molecular unfolding still further. Tanford (*12*) has pointed out that the intrinsic pK_o of these groups in native albumin (10.35 at 25°C) is abnormally high; the typical value for most proteins is near 9.6. Our data indicate that RCA behaves much more like a typical protein in this respect.

The heat and entropy of ionization calculated from the data of Table 2 are in accord with this view. Whereas the phenolic groups of native bovine albumin (*2*) ionize with $\Delta H° = 11.5$ kcal mole^{-1} and $\Delta S° = -8$ entropy units, the corresponding values for RCA (Table 2) are $\Delta H° = 4.3 \pm 1$ kcal mole^{-1} and $\Delta S° = -29 \pm 6$ e.u. Although not highly precise, these values are certainly very different from those of native albumin and reasonably close to those found for tyrosine (*13*) and simple tyrosine peptides (*12*), as would be expected if these groups are freely exposed to the solvent over the whole *p*H range in which they ionize.

The maximum values of $\Delta\varepsilon$, for HSA and RCA, in 0.15*N* NaOH (Table 1) are somewhat lower than might be expected for 18 tyrosyl residues per 69,000 g. For tyrosine $\Delta\varepsilon_{295} = 2400$ and $\Delta\varepsilon_{246} = 11{,}000$ (see Table 1) for ionization of the phenolic group. If we employ these values for HSA and RCA, the data suggest that only 16 of the 18 tyrosyl groups ionize instantaneously. In contrast, a sample of bovine serum mercaptalbumin, prepared in this laboratory by Joel Feigon, showed no such discrepancy; the value of $\Delta\varepsilon_{244} = 225{,}000$ (0.15*N* NaOH vs. *p*H 7) determined for this preparation agreed within the limits of experimental error with the value to be expected for the 21 tyrosyl residues shown to be present by amino acid analysis (*14*). It is also equal to 4.41 times the value of $\Delta\varepsilon_{295} = 51{,}000$, as reported for bovine albumin by Tanford and Roberts (*2*). The factor of 4.41 agrees within the limits of error with our ratio of 4.6 for $\Delta\varepsilon_{244}/\Delta\varepsilon_{295}$ in HSA and RCA.

The spectral changes in acid solutions (*15–17*) also throw important light on structure. The lower part of Table 1 records the change in $\Delta\varepsilon$ on acidification of HSA and RCA. In both cases the difference spectrum shows two prominent peaks at 236 and 287 mμ with a much smaller peak at 279 mμ, in close accord with the data of Glazer and Smith (*3*). The values of $\Delta\varepsilon$ (*p*H 7 − *p*H 1.8) are nearly identical for HSA and RCA, being close to 12,000 at 236 mμ and to 3800 for 287 mμ. For both proteins we found the ratio $\Delta\varepsilon_{236}/\Delta\varepsilon_{287}$ to be constant and equal to 3.30 ± 0.25 in all the solutions studied. For HSA the two $\Delta\varepsilon$ values are plotted as a function of *p*H in Fig. 1 to show the constancy of their ratio. Other investigators (*13–15*) have reported similar curves for absorption at 287 mμ. The blue shift associated with the addition of 8*M* urea to HSA or RCA at *p*H 1.2 (Table 1) likewise gives the same ratio of $\Delta\varepsilon_{236}/\Delta\varepsilon_{287}$.

The constancy of this $\Delta\varepsilon$ ratio provides evidence concerning the origin of the difference peak at 236 mμ. Since the peak at 287 mμ is universally ascribed to changes in the environment of the tyrosine residues, the strict parallelism in the behavior of the two peaks suggests that for serum albumin the peak at 236 mμ is also determined by the surroundings of tyrosine residues. Contributions from other groups, or from changes in the conformation of the peptide chain, would appear to play a negligible role insofar as the difference spectrum is concerned. This conclusion is probably not valid for proteins in general; the work of Glazer and Smith (*3*) indicates that for some other proteins the peak at 236 mμ is probably associated with more general conformational changes in three-dimensional structure (*18*).

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References and Notes

1. R. A. Phelps and F. W. Putnam, in chapter 5, and J. F. Foster, in chapter 6, *The Plasma Proteins*, F. W. Putnam, Ed. (Academic Press, New York, 1960), vol. 1.
2. C. Tanford and G. L. Roberts, Jr., *J. Am. Chem. Soc.* **74**, 2509 (1952).
3. A. N. Glazer and E. L. Smith, *J. Biol. Chem.* **236**, 2942 (1961).
4. E. J. Cohn, L. E. Strong, W. L. Hughes, Jr., D. J. Mulford, J. Ashworth, M. Melin, H. L. Taylor, *J. Am. Chem. Soc.* **68**, 459 (1946).
5. H. M. Dintzis, thesis, Harvard Univ. (1952).
6. B. Jirgensons and I. Ikenaka, *Die Makromolekulare Chem.* **31**, 112 (1959).
7. We take the molecular weight as 69,000 for comparison with the data of Tanford and Roberts (*2*).
8. M. J. Hunter and F. C. McDuffie, *J. Am. Chem. Soc.* **81**, 1400 (1959).
9. E. J. Cohn, W. L. Hughes, Jr., J. H. Weare, *ibid.* **69**, 1753 (1947).
10. F. H. White, Jr., *J. Biol. Chem.* **236**, 1353 (1961).
11. J. Hermans, *Biochemistry* **1**, 193 (1962).
12. C. Tanford, *Advances in Protein Chem.* **17**, 69 (1962).
13. R. B. Martin, D. B. Wetlaufer, B. R. Hollingworth, J. T. Edsall, *J. Biol. Chem.* **233**, 1429 (1958).
14. P. F. Spahr has carried out complete amino acid analyses of human and bovine serum mercaptalbumin.
15. E. J. Williams and J. F. Foster, *J. Am. Chem. Soc.* **81**, 865 (1959).
16. A. N. Glazer, H. A. McKenzie, R. G. Wake, *Biochim. Biophys. Acta* **69**, 240 (1963).
17. T. T. Herskovits and M. Laskowski, Jr., *J. Biol. Chem.* **237**, 2481 (1962).
18. This report is taken in part from the honors thesis of David S. Eisenberg, Harvard 1961. We are indebted to Dr. Margaret J. Hunter for valuable aid and advice in preparing RCA and to Prof. George Wald and P. K. Brown for the use of the Cary spectrophotometer. The HSA was a gift from Dr. R. B. Pennell of the Protein Foundation Laboratories, Jamaica Plain, Mass. Dr. P. F. Spahr made the amino acid analysis. Work was supported by grants from the U.S. Public Health Service (HE 03169) and the National Science Foundation (G9116).

26 August 1963