Takeda, S., Arisaka, F., Ishii, S., & Kyogoku, Y. (1990) *Biochemistry* (preceding paper in this issue).
Tschopp, J., Arisaka, F., van Driel, R., & Engel, J. (1979) *J. Mol. Biol. 128*, 281–286.
Watts, N. R. M., & Coombs, D. H. (1989) *J. Virol. 63*, 2427–2436.
Yamamoto, M., & Uchida, H. (1975) *J. Mol. Biol. 92*, 207–223.

A Comparative Study of the Unfolding Thermodynamics of Vertebrate Metmyoglobins†

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ABSTRACT: Differential scanning microcalorimetry (DSC) of horse, rat, opossum, raccoon, carp, and armadillo metmyoglobins at alkaline pH gave data that fit the two-state unfolding model well. Monte Carlo studies were used to assess the impact of truncating DSC scans on the reliability of the calculated results when aggregation exotherms overlapped the unfolding endotherm at the high-temperature end of the scan. The DSC estimates for the conformational free energy at pH 8 and 298 K are compared to earlier results from isothermal acid and guanidinium chloride unfolding. Stability estimates at pH 8 for these six metmyoglobins obtained by DSC experiments do not agree with free energy estimates at pH 8 from guanidinium chloride unfolding. This is true for all three models used to extrapolate the free energy change to 0 M guanidinium chloride. Among these six myoglobins, significant variation appears in the temperature at which the myoglobin is half-unfolded, in the change in heat capacity upon unfolding, and in the change in enthalpy at 310 K. Calculations made with the hydrophobic model for protein folding [Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069] suggest that a sizable variation exists for that portion of the unfolding enthalpy change assigned to forces other than the hydrophobic effect.

The conformational free energies of the metmyoglobins of at least 16 different species (Puett, 1973; Puett et al., 1973; Pace & Vanderburg, 1979; McLendon, 1977; Bismuto et al., 1984; Holladay, 1985, 1986; Kelly & Holladay, 1987, 1990; Kelly et al., 1988) have been estimated at pH 7–8 and 298 K from guanidinium chloride or urea isothermal studies using the binding models developed by Aune and Tanford (1969). The variation in the estimated stability is large, ranging from 68 kJ/mol for rat and rabbit (Kelly et al., 1988) to 26 kJ/mol for armadillo (Kelly & Holladay, 1990). Other species having myoglobins with especially low stabilities are alligator and snapping turtle (Kelly & Holladay, 1987; Puett et al., 1973). Most mammalian metmyoglobins have an estimated conformational free energy of 38–46 kJ/mol (Puett, 1973; Puett et al., 1973; McLendon, 1977; Flanagan et al., 1983; Bismuto et al., 1984; Kelly & Holladay, 1987, 1990; Kelly et al., 1988). The origin and consequences of these varying stabilities have been the subject of much speculation. Several hypotheses have been advanced as to the selection pressures that may have led to the divergence in stabilities (Puett et al., 1973; Goldberg & Dice, 1974; Goldberg & St. John, 1976; McLendon, 1977; Kelly et al., 1988; Kelly & Holladay, 1990). Some comparative studies (Puett, 1973; Flanagan et al., 1983) have suggested that much of the variation in stability arises from differences in polar and salt bond interactions. Calculations based on Baldwin's (1986) hydrophobic model for protein folding suggest that only a small variation in the change in heat capacity can be tolerated without the loss of the predicted stability. A comparison of the available sequence data and the known tertiary structures of sperm whale (Takano, 1977) and horse (Evans & Brayer, 1988) myoglobins shows that the placement of the hydrophobic internal residues, whose exposure to solvent on unfolding is largely responsible for the change in heat capacity, is well conserved over the course of divergent evolution of these two species.

The thermal denaturation of sperm whale metmyoglobin and apomyoglobin has been extensively studied by Privalov and co-workers (Privalov et al., 1971, 1986; Privalov & Khechinashvili, 1974; Privalov, 1979; Griko et al., 1988) at both alkaline and acid pH. The thermal unfolding is largely reversible if the sample is not heated at too high a temperature for too long a time (Griko et al., 1988). The data obtained suggest that the change in enthalpy on unfolding, once ion binding effects are subtracted out, is dependent only on T_d,[1] the temperature at which the unfolding is half-complete, and not on the pH per se. These data also suggest that the change in heat capacity is, within the precision of the data, reasonably

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[1] Abbreviations: T_d, temperature in kelvin at which unfolding is half-complete; DSC, differential scanning calorimetry; ΔC_p, change in heat capacity on unfolding; $C(T)$, excess heat capacity due to the protein; $C_N(T)$, heat capacity of the native protein; Δn_{H^+}, change in number of bound protons on unfolding; ΔH_{cal}, calorimetric enthalpy change; ΔH_{conf}, conformational enthalpy change; Δn_G, change in number of guanidinium ions bound upon unfolding; $D_{1/2}$, concentration of guanidinium chloride at $\Delta G = 0$; ΔH_{obs}, observed enthalpy change; ΔH_{hyd}, change in enthalpy attributable to the hydrophobic effect; ΔH_{res}, defined as $\Delta H_{obs} - \Delta H_{hyd}$; ΔS_{obs}, observed change in entropy; ΔS_{hyd}, change in entropy attributable to the hydrophobic effect; ΔS_{res}, defined as $\Delta S_{obs} - \Delta S_{hyd}$; ΔG_{obs}, observed change in free energy.

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constant over the interval examined. A crucial finding was that the unfolding process gives the same enthalpy change at either alkaline or acid pH, suggesting that the unfolding process with respect to enthalpy is much the same as any pH.

The observation that the change in heat capacity on unfolding is appreciably less for the apomyoglobin is entirely consistent with previous work on apomyoglobin using other techniques (Crumpton & Polson, 1965; Harrison & Blout, 1965; Breslow et al., 1965; Herskovits & Solli, 1975; Bismuto et al., 1985). The thermal denaturation of horse metmyoglobin (Cho et al., 1982) monitored by the absorbance at the Soret band and analyzed by the van't Hoff equation suggests that the unfolding enthalpy change is essentially identical for the aquomet, hydroxymet, and cyanomet derivatives. The cyanomet form appears to be about 12 kJ/mol more stable than the hydroxymet form (Cho et al., 1982). The enhanced stability of the cyanomet form demonstrates that a substantial alteration in stability can result even though the enthalpy change remains the same.

These observations suggest that a comparative calorimetric study of the thermal denaturation of myoglobins of divergent stabilities would be of some interest. This study examines the thermal denaturation of armadillo, horse, rat, opossum, raccoon, and carp metmyoglobin at alkaline pH. The free energy of unfolding of these metmyoglobins has previously been estimated at pH 8, 25 °C, from guanidinium chloride and urea isothermal unfolding data (Puett, 1973; Holladay, 1986; Kelly et al., 1988; Kelly & Holladay, 1990). The calorimetric data are used to directly estimate the conformational free energy at pH 8, 298 K, allowing a comparison with the extrapolation models for isothermal denaturant data. In addition, a comparison of the temperature dependence of the unfolding enthalpy change shows to what degree the observed differences in stabilities result from divergent evolution of nonpolar contacts. Finally, the data are analyzed according to Baldwin's (1986) hydrophobic model for protein folding to estimate what portion of the unfolding enthalpy change for each species results from hydrophobic forces.

Materials and Methods

Horse skeletal muscle myoglobin was a commercial product. Other salts and common reagents were analytical grade. Purified water with a conductivity of less than 60 $\mu\Omega^{-1}$ was used.

Carp, rat (peak II), opossum, raccoon, and armadillo metmyoglobins were purified as described previously (Holladay, 1986; Kelly et al., 1988; Kelly & Holladay, 1990). Horse skeletal muscle metmyoglobin was dissolved in a small amount of water, oxidized to the met form by slow addition in the cold of $K_3Fe(CN)_6$, and exhaustively dialyzed against water and then against 7 mM pH 7.95 Tris-HCl. The horse metmyoglobin was purified on a CM-Sephadex column as described earlier (Puett, 1973). The pooled peak was dialyzed against water, and aliquots were frozen at −38 °C.

Metmyoglobin solutions were prepared by dissolving the lyophilized carp, rat, opossum, raccoon, or armadillo protein in either 10 mM piperazine hydrochloride or 100 mM sodium glycinate at a concentration of about 4 mg/mL and dialyzing for 2 h at 25 °C against 50 mL of the same buffer at the desired pH. Horse metmyoglobin in water at a concentration of about 3.6 mg/mL was dialyzed against the desired buffer. To obtain the cyanomet form of horse metmyoglobin, 5 mM NaCN was added to the buffers. After dialysis, the pH of the solution and of the dialyzate was measured.

The concentration of the protein in the retentate was estimated from the absorbance at 280 nm. The extinction coefficient increases slightly as the pH increases (Hanania et al., 1966). The extinction coefficients at 280 nm for these metmyoglobins were determined at alkaline pH by the pyridine hemochromogen method (deDuve, 1948). The extinction coefficient at 280 nm of horse metmyoglobin was found to be essentially identical for both the cyanomet and hydroxymet forms at alkaline pH. For both horse and armadillo myoglobins the dependence of 280-nm absorbance on pH was experimentally determined and was used to calculate an extinction coefficient as a function of pH. Above pH 9 the extinction coefficient changed little, and for other myoglobins the extinction coefficient at 280 nm was determined at pH 9 and assumed to be essentially unchanged from pH 9 to pH 12.

A Hart Scientific differential scanning calorimeter was used to carry out thermal unfolding studies. Samples of close to 0.5-mL volume contained about 2 mg of protein. The heating rate was 1 deg/min. Reversal studies were done by heating the solution to a temperature at which the protein was at least nine-tenths unfolded and cooling to 20 °C at a rate of 1.5 deg/min, staying near 20 °C for at least 30 min, and rescanning.

The calorimetry data were fit with the Simplex algorithm (Nelder & Mead, 1965; Cacece & Cacheris, 1984) to an expression derivable from the van't Hoff relationship. Although over a long temperature interval the change in heat capacity ΔC_p cannot be constant (Privalov & Gill, 1989), over the short temperature interval of protein unfolding the heat capacity change should be sufficiently constant (Privalov, 1979) to allow for data analysis with this model. The equation used was

$$C(T) = C_N(T) + e^U \Delta C_p/(e^U + 1) + (e^U/R)([\Delta H + \Delta C_p(T - T_d)]/[T(e^U + 1)])^2$$

where

$$U = [(\Delta C_p T_d - \Delta H)(1/T - 1/T_d) + \Delta C_p \ln (T/T_d)]/R$$

and ΔH is at $T = T_d$. Here, $C_N(T)$, the heat capacity of the native protein, is assumed to be linear in temperature and equal to $C_N(310\text{ K})$ + slope × $(T - 310\text{ K})$. This model implies that the heat capacity of the unfolded protein is also a linear function of temperature. The value for ΔC_p was constrained to be nonnegative. This was accomplished by rewriting the above equation so that ΔC_p was replaced by a new parameter, the square of the square root of ΔC_p. The substitute parameter (the square root of ΔC_p) is free to be either positive or negative. The resulting value of ΔC_p obtained by squaring the substitute parameter ensures that ΔC_p is nonnegative.

The values for ΔH, T_d, and ΔC_p were returned for each run from the heat absorbed and temperature raw data fit to the above $C(T)$ equation with the Simplex algorithm. The values of T_d extrapolated to pH 8 by a least-squares parabolic fit and ΔH extrapolated to T_d at pH 8 by a linear least-squares regression were then obtained for each species. The value for ΔC_p for each species was taken to be the slope of the ΔH vs T_d regression line. This estimate for ΔC_p is more reliable than that obtained from nonlinear regression analysis of individual unfolding curves. The value for ΔG at any temperature T can then be calculated from the estimated values of ΔH at pH 8 at T_d, ΔC_p, and T_d from

$$\Delta G_T = \Delta H[(T_d - T)/T_d] + \Delta C_p(T - T_d) - T\Delta C_p \ln (T/T_d)$$

This equation holds provided that ΔC_p is constant over the temperature interval between T and T_d (Privalov & Khechinashvili, 1974).

The change in the number of bound protons Δn_{H^+} was calculated from

$$\Delta n_{H^+} = (\Delta H_{cal}/2.3RT^2)(dT_d/d\,pH)$$

where $dT_d/d\,pH$ was estimated by fitting the T_d vs pH data to a second-order polynomial in T_d and ΔH_{cal} is the calorimetric enthalpy change.

A set of approximate 95% confidence intervals for T_d, ΔH, and ΔC_p were obtained by examining the residual sum of squares surface along each parameter axis, holding all other parameters at their best values. The ends of the confidence intervals were taken as the parameter values giving a sum of squared residuals for which the lack of fit of the $F(p,n-p)$ test just exceeded the 0.95 value (Draper & Smith, 1966). For several sets of data all ten cross sections of the six-dimensional residual sum of squares surface were calculated as contour plots and examined.

To ascertain just how approximate the approximate confidence intervals are, a Monte Carlo analysis was done on a parameter set and experimental noise typical of our data. Twenty-five data sets with identical parameters [280 data points ranged from 310 to 360 K and were generated with T_d set at 342 K, ΔH set equal to 440 kJ, and ΔC_p set at 8.0 kJ/(mol K)] and differing sets of normally distributed noise were analyzed in the same way as for collected data described above. The Monte Carlo analysis was repeated for 25 data sets that were truncated on the descending portion of the unfolding curve to observe the effect of discarding data on the tail of the unfolding endotherm influenced by aggregation or insolubility effects of the unfolded protein.

Results

The extinction coefficient at neutral pH and 280 nm for horse metmyoglobin is 29 100, 1500 less than that for sperm whale metmyoglobin (Hapner et al., 1968). This difference probably arises because the sperm whale protein has an additional tyrosyl residue at position 151. An additional buried tyrosyl residue is expected from model compound data to increase the extinction coefficient by about this amount (Bailey, 1966). At pH 9, the extinction coefficient for horse increases to 30 900. Also determined at pH 9 are extinction coefficients for armadillo (32 200), rat (31 600), opossum (28 600), raccoon (33 200), and carp (25 900). The observed differences in extinction coefficients for the species studied here are probably largely due to differing tyrosyl (and in the case of carp differing tryptophanyl) content.

Separate confidence interval estimates for T_d, ΔC_p, and ΔH were estimated from Monte Carlo studies of noisy-simulated DSC data. For each of these three parameters, the approximate 95% separate confidence intervals did not vary appreciably between data sets. For T_d, the approximate 95% confidence interval estimated from a single data set did not differ greatly from the range that enclosed 92% of the 25 estimates. Thus, for T_d, the approximate 95% separate confidence interval is a conservative estimate of the probable error. For ΔH at T_d, this procedure appears to overestimate the probable error by a factor of about 2. This procedure underestimates by a factor of 2–3 the likely error in ΔC_p, perhaps due to the nonlinearity of the model with respect to this parameter (Beale, 1960; Guttman & Meeter, 1965).

The two parameters of principal interest, ΔH and T_d, show no appreciable correlation with each other nor with ΔC_p or the two parameters characterizing the heat capacity of the native protein. The other parameter of interest, ΔC_p, shows a considerable correlation with the parameters characterizing $C_N(T)$, the heat capacity of the native protein.

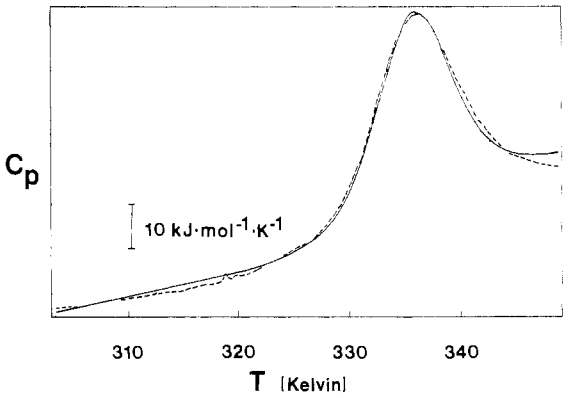

FIGURE 1: Calorimetric scan (dashed line) of horse metmyoglobin at pH 11.15 (25 °C), 100 mM sodium glycinate buffer. The solid curve is calculated from the parameters in Table II. Reversibility of unfolding of horse cyano- or hydroxy-metmyoglobin was similar, ranging from 74% at high pH to 91% at lower pH.

As the pH of the solutions decreased toward neutral, at some point the unfolding endotherms of all the metmyoglobins were followed by a large overlapping exotherm, presumably arising from irreversible aggregation. For a few data sets only an estimate for T_d was obtainable. For all the metmyoglobins, data were collected from pH 12 down to the lowest pH giving analyzable data. Some of the data sets for which the unfolding endotherm was followed later by an aggregation exotherm were truncated at a point for which the unfolding endotherm was 70–80% complete in order to discard the region in which the overlap occurred.

A repeated analysis was done to show the effects on the confidence intervals for the three thermodynamic parameters of interest obtained from truncated data sets. For ΔH with the truncated simulated data sets, the approximate 95% separate confidence interval turned out to enclose only about 50% of the returned estimates.

For T_d with the truncated simulated data sets, the approximate 95% separate confidence interval enclosed only about 50% of the returned estimates. For the more complete data sets, the approximate 95% separate confidence interval enclosed at least 95% of the returned estimates. For ΔC_p, for both the complete and truncated data sets, the approximate 95% confidence intervals enclosed between 40 and 60% of the returned estimates.

Figure 1 shows a sample DSC scan for horse metmyoglobin. The line drawn in Figure 1 is the theoretical curve for the two-state model calculated from the parameter estimates obtained from DSC data and shown in Table I. The fits of the data to the two-state model were generally quite good for all the metmyoglobins studied, with the lack of fit on the order of the instrumental noise. Reversibilities of unfolding of horse cyanomet- or hydroxymet-myoglobins were similar and ranged from 74% at pH near 12 to 91% at lower pH. If the horse myoglobin solutions were heated to temperatures substantially higher than required to complete the transition, reversibility was quite poor. In general, the higher the temperature reached and the higher the pH, the poorer the reversibility of horse metmyoglobin unfolding.

The DSC scan for armadillo metmyoglobin unfolding is shown in Figure 2. Table II gives the parameters similarly obtained at several pH values for armadillo hydroxymet- and aquometmyoglobins. Armadillo metmyoglobin unfolding reversal studies gave complex DSC scans with multiple peaks at both acid and alkaline pH. Figure 2 also shows a refolding reversal scan in which the solution was kept for 30 min at 20 °C before being rescanned. If the solution is kept for longer

Table I: Summary of Scanning Calorimetric Data for Horse Metmyoglobin

pH (25 °C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
Hydroxymetmyoglobin (100 mM Sodium Glycinate)					
11.77	326.9[a]	344 ± 4[c]	4.6–5.4	1.0[d]	−2.1
11.69	326.2[b]	345 ± 2	7.2–7.6	0.5	−2.1
11.55	330.8[a]	377 ± 2	2.4–3.0	0.5	−2.1
11.15	335.2[b]	409 ± 4	12.1–13.1	1.1	−2.0
11.15	336.5[b]	413 ± 3	7.8–8.3	0.7	−2.0
10.76	339.7[a]	432 ± 3	9.8–10.8	0.8	−1.9
10.34	345.9[b]	498 ± 7	2.0–4.3	2.2	−1.9
10.14	347.7[a]	496 ± 4	6.9–8.0	1.0	−1.7
9.87	349.9[b]	528 ± 5	10.2–11.6	1.6	−1.7
9.44	354.2	(overlapping aggregation exotherm)			
Cyanometmyoglobin (100 mM Sodium Glycinate, 5 mM NaCN)					
11.80	335.9[c]	455 ± 4	10.6–11.3	1.2	[e]
10.87	348.7[a]	539 ± 4	9.6–11.1	1.2	[e]
9.91	360.0[b]	603 ± 8	7.4–11.8	2.5	[e]

[a] The data set terminated within 5–10 °C above T_d. [b] The data set terminated within 11–15 °C above T_d. [c] Approximate 95% confidence interval. [d] Root mean square residual lack of fit for data set. [e] Not enough data to calculate.

Table II: Summary of Scanning Colorimetric Data for Armadillo Metmyoglobin

pH (25 °C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
Hydroxymetmyoglobin (100 mM Sodium Glycinate)					
11.20	332.6[a]	329 ± 4[e]	0	1.0[f]	−1.7
10.83	337.3[a]	362 ± 2	5–7	0.5	−1.6
10.72	338.4[a]	374 ± 5	0	1.2	−1.6
10.72	339.6[b]	392 ± 4	0	1.0	−1.7
10.32	342.6[a]	383 ± 5	0	1.5	−1.4
10.01	346.6[b]	382 ± 8	6–8	2.2	−1.3
9.90	346.7[d]	406 ± 4	4	2.0	−1.3
9.90	347.0[d]	397 ± 6	0	1.7	−1.3
Aquometmyoglobin (10 mM Piperazine Hydrochloride)					
5.49	345.7[c]	387 ± 3	4.4–5.0	0.8	6.6
5.49	346.5[a]	374 ± 2	0	0.6	6.4
5.42	343.9[d]	392 ± 5	4.1–4.6	1.6	6.6
5.33	340.8[a]	302 ± 3	2.9–3.8	0.7	5.0
5.14	337.7[a]	291 ± 2	0	0.5	4.4
4.92	332.5[d]	277 ± 4	4.4–4.9	0.9	3.8

[a] The data set terminated within 2–5 °C above T_d. [b] The data set terminated within 6–10 °C above T_d. [c] The data set terminated within 11–15 °C above T_d. [d] The data set terminated within 16–33 °C above T_d. [e] Approximate 95% confidence interval. [f] Root mean square residual lack of fit for data set.

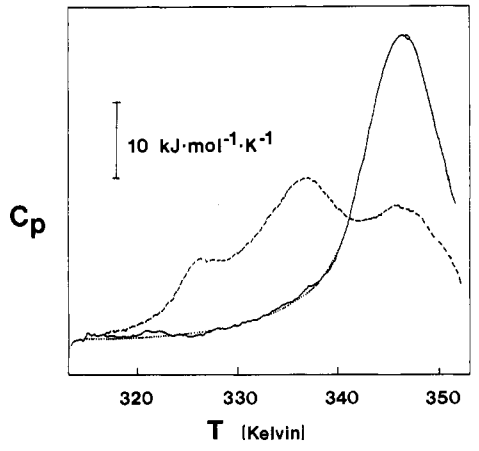

FIGURE 2: Calorimetric scan (solid curve) of armadillo metmyoglobin at pH 5.5 (25 °C), 10 mM piperazine hydrochloride buffer. The dotted curve is the theoretical curve calculated from the data of Table I. The dashed line is the calorimetric scan resulting after refolding for 30 min at 25 °C at pH 5.5 after heating to 350 K. If the refolding time is extended to 2 h, the peak with the intermediate T_d decreases, and the peak with the native T_d increases (data not shown).

Table III: Summary of Scanning Calorimetric Data for Rat Hydroxymetmyoglobin in 100 mM Sodium Glycinate

pH (25 °C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
11.49	331.0[c]	307 ± 6[d]	13.5–14.0	2.6[e]	−1.5
11.18	333.8[a]	344 ± 3	0	0.7	−1.7
10.92	338.6[b]	378 ± 3	0	0.8	−1.7
10.66	341.2[a]	365 ± 6	0	1.5	−1.6
10.55	341.8[c]	444 ± 9	9.1–11.0	2.6	−1.9
10.39	344.3[c]	435 ± 4	5.5–6.4	1.2	−1.9
10.00	349.7[c]	462 ± 8	2.9–4.7	2.6	−1.8
9.56	355.1[a]	441 ± 9	0–7.7	2.6	−1.6
9.13	357.9[a]	503 ± 8	0	2.3	−1.8

[a] The data set terminated within 5 °C above T_d. [b] The data set terminated within 6–10 °C above T_d. [c] The data set terminated within 11–15 °C above T_d. [d] Approximate 95% confidence interval. [e] Root mean square residual lack of fit for data set.

Table IV: Summary of Scanning Calorimetric Data for Opossum Hydroxymetmyoglobin in 100 mM Sodium Glycinate

pH (25 °C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
11.50	330.3[d]	307 ± 4[e]	15–16	0.9[f]	−1.4
11.50	332.2[a]	333 ± 3	5.7–6.8	0.6	−1.5
11.19	333.5[b]	368 ± 4	9–10	1.2	−1.6
10.97	336.2[d]	359 ± 3	7.3–7.9	0.9	−1.5
10.65	340.9[a]	374 ± 2	0–1	0.5	−1.5
10.38	343.9[a]	377 ± 5	0	1.3	−1.4
9.98	346.9[d]	436 ± 6	0	2.0	−1.5
9.63	349.9[a]	430 ± 6	0	1.5	−1.4
9.52	351.2	(overlapping aggregation exotherm)			

[a] The data set terminated within 2–5 °C above T_d. [b] The data set terminated within 6–10 °C above T_d. [c] The data set terminated within 11–15 °C above T_d. [d] The data set terminated within 16–23 °C above T_d. [e] Approximate 95% confidence interval. [f] Root mean square residual lack of fit for data set.

Table V: Summary of Scanning Calorimetric Data for Raccoon Hydroxymetmyoglobin in 100 mM Sodium Glycinate

pH (25 °C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
11.96	323.0[d]	249 ± 3[e]	8–9	0.5[f]	−1.9
11.92	325.5[b]	293 ± 3	9–10	0.7	−2.2
11.49	335.0[b]	331 ± 4	17–18	0.9	−2.0
11.10	338.0[a]	364 ± 4	10–12	1.0	−1.9
11.09	340.7[c]	413 ± 6	14–15	3.3	−2.0
10.87	343.4[b]	415 ± 3	8–9	0.9	−1.8
10.59	345.8[d]	412 ± 4	4–5	1.0	−1.6
10.37	348.0[b]	440 ± 3	4–5	0.8	−1.5
10.18	349.0[c]	437 ± 7	0	1.9	−1.3
9.99	352.3[a]	446 ± 6	0	1.8	−1.2

[a] The data set terminated within 3–5 °C above T_d. [b] The data set terminated within 6–10 °C above T_d. [c] The data set terminated withtin 11–15 °C above T_d. [d] The data set terminated within 16–22 °C above T_d. [e] Approximate 95% confidence interval. [f] Root mean square residual lack of fit for data set.

times at the same temperature before being rescanned, the heights of the two endotherms with lower T_d decrease, and the endotherm with the T_d of the native protein increases. At alkaline pH two endotherms are seen on refolding after 1 h at pH 10.83, one with a T_d identical with that of the native protein and one with a T_d about 10 °C lower.

Tables III–VI summarize the thermodynamic parameters at the indicated alkaline pH values obtained from DSC data for rat, opossum, raccoon, and carp hydroxymetmyoglobins. Rat metmyoglobin unfolding was 81% reversible at pH 11.5. Opossum metmyoglobin unfolding was 93% reversible at pH 11.5. Raccoon metmyoglobin unfolding was 93% reversible at pH 11.1. With these four metmyoglobins only a single endotherm was seen in the reversal scans. Carp metmyoglobin, which has a cysteinyl at position 13 (Romero-Herrera et al.,

Table VI: Summary of Scanning Calorimetric Data for Carp Hydroxymetmyoglobin in 100 mM Sodium Glycinate

pH (25° C)	T_d (K)	ΔH_{cal} (kJ/mol)	ΔC_p [kJ/ (mol K)]	$s(C_p)$ [kJ/ (mol K)]	Δn_{H^+}
11.97	311.0[d]	158 ± 3[e]	2–3	0.6[f]	−1.2
11.85	311.0[c]	181 ± 3	1	0.5	−1.4
11.85	312.1[b]	158 ± 2	2	0.5	−1.2
11.60	317.6[d]	186 ± 3	0–1	0.7	−1.2
11.27	323.8[b]	241 ± 3	0	0.6	−1.3
10.97	327.8[d]	301 ± 5	0	1.5	−1.4
10.87	327.9[c]	292 ± 4	2–3	1.0	−1.4
10.71	328.9[d]	326 ± 7	0	2.4	−1.4
10.55	331.2[b]	290 ± 2	1–2	0.5	−1.2
10.36	333.2[a]	306 ± 4	0	1.1	−1.1
10.15	335.6[a]	344 ± 3	0–2	0.8	−1.1
9.98	337.2[a]	380 ± 4	0	1.0	−1.1
9.94	336.4[c]	341 ± 6	4–6	1.6	−1.0

[a] The data set terminated within 2–5 °C above T_d. [b] The data set terminated within 6–10 °C above T_d. [c] The data set terminated within 11–15 °C above T_d. [d] The data set terminated within 16–25 °C above T_d. [e] Approximate 95% confidence interval. [f] Root mean square residual lack of fit for data set.

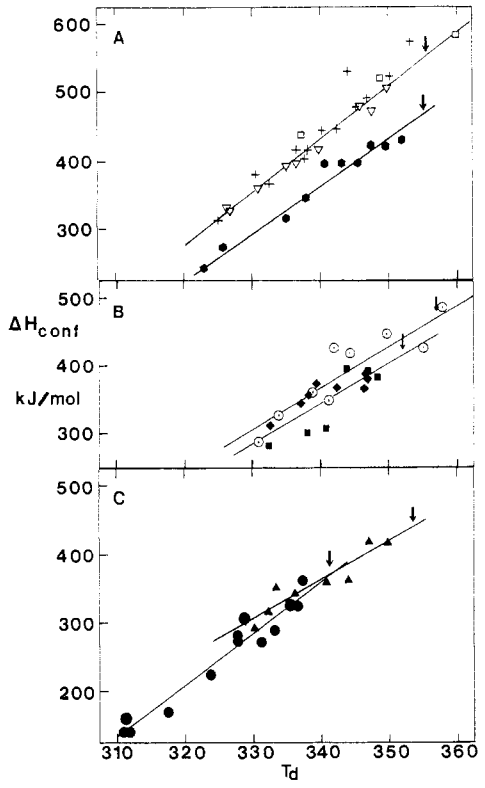

FIGURE 3: Conformational enthalpy change for mammalian metmyoglobins plotted as a function of temperature: (Panel A) (Open down triangles) Horse hydroxymet; (open squares) horse cyanomet at alkaline pH; (crosses) sperm whale aquometmyoglobin data from Privalov et al. (1986); (hexagons) raccoon hydroxymet. (Panel B) (Diamonds) Armadillo hydroxymet; (filled squares) armadillo aquomet; (open circles) rat hydroxymet. (Panel C) (Filled triangles) Opossum hydroxymet; (filled circles) carp hydroxymet. The least-squares lines were calculated with only the data obtained in this study. Arrows indicate extrapolated T_d's at pH 8.

1982), showed essentially no reversibility of unfolding. This is very likely due to the formation of disulfide dimers of the unfolded globin at elevated temperature and pH. Even for irreversible denaturation the van't Hoff relationship may provide a reliable method for data analysis provided that a reasonably rapid reversible equilibrium between native and unfolded forms is followed by a slower irreversible step (Edge et al., 1985).

Calculation of the conformational enthalpy changes shown in Figure 3 from the calorimetric enthalpy changes at alkaline

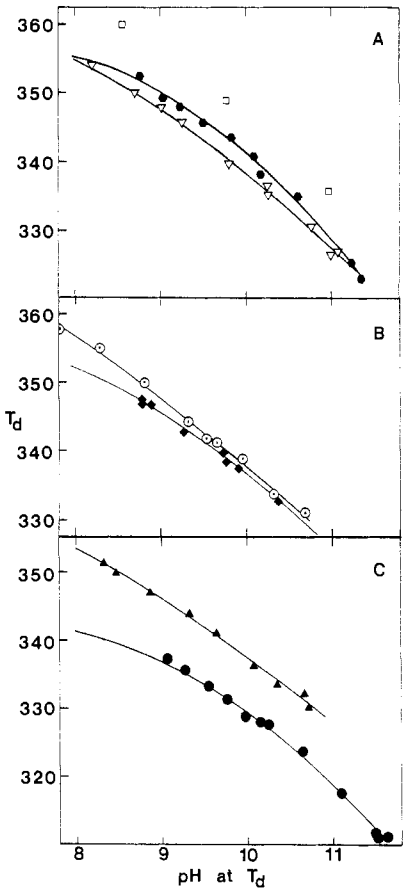

FIGURE 4: Unfolding midpoint temperature T_d plotted against the pH at that temperature: (Panel A) (Open down triangles) Horse hydroxymet; (open squares) horse cyanomet at alkaline pH; (hexagons) raccoon hydroxymet. (Panel B) (Filled squares) Armadillo aquomet; (open circles) rat hydroxymet. (Panel C) (Filled triangles) Opossum hydroxymet; (filled circles) carp hydroxymet. The curves shown represent least-squares parabolic fits.

pH given in Tables I–VI requires a small correction that depends upon the knowledge of the number of buried tyrosyls (Privalov et al., 1986). The tyrosyl at position 146 is buried in both sperm whale (Takano, 1977) and horse (Evans & Brayer, 1988) myoglobin. It is known that, upon unfolding of sperm whale myoglobin, one tyrosyl is exposed (Breslow, 1964). For vertebrate myoglobins that have been sequenced, position 146 is invariably tyrosyl. The tyrosyls at the other positions in sperm whale and horse myoglobins are not buried. For all hydroxymetmyoglobins, ΔH_{cal} was corrected for ion binding, assuming that one tyrosyl residue becomes ionized on unfolding and that four histidyl residues become protonated for armadillo aquometmyoglobin on unfolding (Kelly & Holladay, 1990). Figure 3 shows the resulting conformational enthalpy change on unfolding, ΔH_{conf}, for the metmyoglobins as a function of temperature. Figure 4 shows the dependence of T_d on pH calculated at T_d.

For all the species studied, the data are summarized in Table VII and compared to previous results obtained by isothermal denaturant studies at pH 8 with the data analyzed by either the transfer model of Tanford as expanded by Puett (Tanford, 1964; Puett, 1972), the binding model of Aune and Tanford (1969), or the linear extrapolation model (Schellman, 1978, 1987; Schellman & Hawkes, 1980). The binding model of Aune and Tanford gives estimates for the conformational free energy which are very close to those of the transfer model (Puett, 1973; Holladay, 1985, 1986; Kelly & Holladay, 1987, 1990; Kelly et al., 1988). From the regression line for ΔH_{conf} vs T and the estimate for T_d at pH 8, ΔH at T_d (pH 8) was

Table VII: Comparison of DSC and Denaturant Studies at pH 8[a]

species	ΔG (transfer model)	$\Delta\alpha$	Denaturant Data ΔG if $\Delta\alpha$ = 0.25	ΔG (binding model)[b]	Δn	ΔG (linear model)	$D_{1/2}$ (mol/L)
rat[c]	67	0.36	46	66	64	39	1.84
raccoon[c]	64	0.36	44	62	64	37	1.69
horse	43[d]	0.25[d]	43[d]	40[e]	45[e]	25[d]	1.63[d]
opossum[c]	54	0.31	43	52	55	31	1.61
armadillo[f]	27	0.16	41	26	29	16	1.46
carp[g]	38	0.24	40	37	40	27	1.62

species	T_d	DSC Data ΔH (at T_d)	ΔC_p	ΔG(298 K)
rat	357.0 ± 0.6	473 ± 27	6.2 ± 1.0	46 ± 5
raccoon	355.3 ± 0.9	465 ± 16	6.8 ± 0.5	42 ± 3
horse	354.7 ± 0.9	548 ± 20	7.8 ± 0.5	50 ± 3
opossum	353.5 ± 0.7	435 ± 18	5.6 ± 0.9	43 ± 6
armadillo	352.0 ± 0.5	417 ± 24	6.0 ± 1.3	38 ± 6
carp	341.3 ± 0.7	370 ± 19	7.6 ± 0.6	25 ± 3

[a] ΔG and ΔH are in kJ/mol, ΔC_p is in kJ/(mol K), and ΔG is at 298 K. [b] Using a binding constant of 0.6 (Pace & Vanderburg, 1979). [c] Kelly et al., 1988. [d] Puett, 1973. [e] Data at pH 6 (Pace & Vanderburg, 1979). [f] Kelly & Holladay, 1990. [g] Holladay, 1986.

estimated. From T_d, ΔH at T_d, and ΔC_p, an estimate for ΔG at 298 K is calculable (Privalov, 1979). The probable error in ΔG was estimated by varying the first three parameters over a one standard deviation range.

Discussion

In addition to the calculation of thermodynamic parameters from the heat absorbed during metmyoglobin unfolding, this study has addressed the reliability with which those estimates can be endowed. Unfortunately, not all data sets from DSC have an extensive base line characteristic of the heat capacity of the unfolded species trailing the unfolding endotherm. The Monte Carlo analysis of abbreviated data sets indicates the estimates for both ΔH and ΔC_p get somewhat less reliable as the temperature range above T_d is contracted. We were surprised to discover that the degree to which "approximate 95%" varied from 95% depends on the completeness of the data set analyzed. The root mean square lack of fit for the plots of ΔH_{conf} vs T_d varied from 16 to 27 kJ/mol. Thus, the error in determining ΔH is about 20 kJ/mol, considerably larger than that suggested by the smaller approximate 95% confidence intervals. These results point out that, for parameters in nonlinear regression models, the approximate confidence intervals can be very approximate indeed. Moreover, the degree to which these intervals are approximate depends upon the data set as well as the model.

Previously, the conformational free energies of horse, rat, opossum, raccoon, carp, and armadillo metmyoglobins were estimated from guanidinium chloride, urea, and acid unfolding studies (Puett, 1973; Pace & Vanderburg, 1979; Holladay, 1986; Kelly et al., 1988; Kelly & Holladay, 1987, 1990). When only the mammalian myoglobins are considered, there is an excellent correlation between the denaturant concentration required to unfold half the myoglobin and the estimate for T_d at pH 8. Thus, the ordering of stability, from most to least stable, is fairly robust in that the same ordering is given by the two methodologies for a series of mammalian myoglobins which do not differ too greatly in sequence. Carp metmyoglobin is at most about 40% homologous to mammalian myoglobins (Romero-Herrera et al., 1982) and has a value for T_d at pH 8 at least 10 °C lower than that of the mammalian metmyoglobins. Although the T_d and the denaturant concentration required for unfolding of half of the molecules are not the same as thermodynamic differences between native and unfolded states, it may be that comparisons of the former two observations will not be meaningful if substantial differences exist between myoglobin unfolded by heat and myoglobin unfolded by guanidinium chloride.

A striking disparity between denaturant and the thermal unfolding stability estimates can result from even a single amino acid residue change for cytochrome *c*. Rat cytochrome *c* can be stabilized by about 1.5 kcal/mol at 25 °C when tyrosine-67 is substituted with phenylalanine as measured by thermal titration of the absorbance at 695 nm (Luntz et al., 1989). However, no significant difference in susceptibility to unfolding by urea could be detected when monitored by absorption at 695 nm. For the native and substituted cytochrome *c* pair, a difference of 30 °C in $t_{1/2}$ (T_d) is coupled with at most an insignificant 0.1 M difference in $D_{1/2}$ for urea denaturation.

With either denaturant or calorimetric data, a long and hazardous extrapolation is required to estimate the conformational free energy at room (or body) temperature at neutral pH (Pfeil, 1981). In extrapolation of calorimetric data back to lower temperatures, the uncertainties in both ΔC_p and the estimated enthalpy change at T_d (at pH 8) are major factors limiting the reliability of the extrapolation. In the extrapolation of denaturant data, the major difficulties are the choice of the extrapolation model and the variation from protein to protein of the parameter characterizing the dependence of the free energy change on denaturant. A central problem in comparing the estimates obtained for the conformational free energy from calorimetry and denaturant data is that it is not certain than the denatured states of protein molecules obtained with these two techniques are identical with respect to their thermodynamic properties.

The most puzzling aspect of the metmyoglobin denaturant studies is the wide variation in the apparent cooperativity of the unfolding process with respect to denaturant concentration. The transfer model parameter $\Delta\alpha$ varies from 0.16 (Kelly & Holladay, 1990) to 0.36 (Kelly et al., 1988). This parameter is supposed to represent the difference in exposure to the solvent for the average side chain and peptide group upon unfolding (Tanford, 1964). A simplistic interpretation of the high values of 0.36 found for $\Delta\alpha$ for raccoon and rat metmyoglobins is that these two species have more compact myoglobins. Horse and sperm whale have very similar tertiary structures (Takano, 1977; Evans & Brayer, 1988) and $\Delta\alpha$ values of 0.25 (Puett, 1973). It does not seem likely that the folding pattern of rat and raccoon myoglobins is that much more compact than that for horse and sperm whale. If the experimentally determined $\Delta\alpha$ values are used in the analysis, the agreement between transfer model estimates for the conformational free energy with those calculated from the DSC data is poor (Table VII). If a common pooled value of 0.25

Table VIII: Hydrophobic Folding Model Calculations at 310 K[a]

species	ΔH_{obs}	ΔH_{hyd}	ΔH_{res}	ΔS_{obs}	ΔS_{hyd}	ΔS_{res}	ΔG_{obs}
rat	180	90	90	0.45	−1.4	1.8	42
raccoon	160	100	60	0.39	−1.5	1.9	39
horse	200	120	80	0.50	−1.7	2.2	46
opossum	190	80	110	0.50	−1.2	1.7	38
armadillo	160	90	70	0.42	−1.3	1.7	34
carp	130	110	20	0.35	−1.7	2.0	23

[a] ΔH_{obs}, ΔH_{hyd}, ΔH_{res}, and ΔG_{obs} are in kJ/mol; ΔS_{obs}, ΔS_{hyd}, and ΔS_{res} are in kJ/(mol K).

is used for $\Delta\alpha$, the agreement between the transfer model and the DSC data improves somewhat, with carp metmyoglobin providing a major discrepancy. One possible problem with the transfer model is that there well may be considerable variation in the burial of classes of side chains. As a result, the averaging of all the individual $\Delta\alpha_i$ into one lumped term may lead to wide variation in the average $\Delta\alpha$ which does not correctly reflect the actual transfer free energies. It is puzzling that no correlation exists between ΔC_p and $\Delta\alpha$ even though these two parameters are believed to arise largely from the same phenomenon, the burial of the hydrophobic side chains.

For horse (Puett, 1973), carp (Holladay, 1986), armadillo (Kelly & Holladay, 1987), and alligator (Kelly & Holladay, 1990) metmyoglobins, acid unfolding data at low guanidinium chloride concentrations are available. When these data are analyzed by the binding model of Aune and Tanford (1969), the resulting parameters for the conformational free energy and the increase in the number of bound guanidinium ions are not significantly different from those obtained from pH 8 data with much higher denaturant concentrations (Puett, 1973; Pace & Vanderburg, 1979; Holladay, 1986; Kelly & Holladay, 1987, 1990). It may well be that the binding constant is constant neither over the range of denaturant concentrations used nor over the range of species of metmyoglobins examined. It is well-known that the fit of experimental data is quite insensitive to the value used for the binding constant and that different values of the binding constant yield differing estimates for the conformational free energy.

While the linear extrapolation model has received some justification and use (Santoro & Bolen, 1988; Schellman, 1978, 1987; Schellman & Hawkes, 1980; Shortle et al., 1989), little evidence exists that the estimates this model yields are more accurate than those of the binding and transfer models. Our data suggest that for the myoglobins neither the linear model, the transfer model, nor the binding model yields estimates for the conformational free energy comparable to those obtained from calorimetry data. This lack of agreement may arise from either the nonequivalency of unfolded states or the inadequacy of the simplistic models used for the analysis of denaturant data.

The apparent nonparallelism among the least-squares lines describing the variation of enthalpy change with temperature for these myoglobins suggests that there is some variation in the change in heat capacities on unfolding of these myoglobins. Thus, these data suggest only partial conservation of internal nonpolar contacts over the course of vertebrate evolution. There is a considerable degree of conservation of the internal residues as revealed from the known vertebrate myoglobin sequences, but there well may be some variation in the tertiary structures as a result of 0.5 billion years of divergent evolution. Recent studies point out that when an internal residue is substituted due to an evolutionary process, the net effect upon the stability of the protein depends upon the energetics of two considerations. One change is in any packing effects upon the internal structure of the molecule due to the altered residue size; the other change is in the hydrophobicity of the internal residues (Sandberg & Terwilliger, 1989).

Table VIII shows the DSC data analyzed with Baldwin's hydrophobic model for protein folding at 310 K (Baldwin, 1986). ΔH_{hyd}, the hydrophobic contribution to the observed enthalpy change, does not vary greatly, with the major variation in the observed enthalpy change due to ΔH_{res}, the residual enthalpy change, which is presumably due to polar interactions. If these data and their analysis in terms of the hydrophobic model are correct, the residual entropy change for all the myoglobins should be very similar, assuming that the native structures are similar and that the unfolded forms of all the myoglobins are equally disordered. The calculated residual entropy changes at 310 K are probably identical within experimental error.

Supplementary Material Available

A table presenting the Monte Carlo study of the analysis of noisy simulated DSC data showing the results of analysis of 25 data sets with normally distributed random noise followed by analysis of 25 sets of truncated data (4 pages). Ordering information is given on any current masthead page.

References

Aune, K. C., & Tanford, C. (1969) *Biochemistry 11*, 4586.
Bailey, J. E. (1966) Ph.D. Thesis, London University.
Baldwin, R. L. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8069.
Beale, E. M. L. (1960) *J. R. Statist. Soc. Ser. B22*, 41.
Bismuto, E., Irace, G., Servillo, L., Giovane, A., & Colonna, G. (1974) *Experentia 40*, 1400.
Bismuto, E., Colonna, G., Savy, F., & Irace, G. (1985) *Int. J. Pept. Protein Res. 26*, 195.
Breslow, E. (1964) *J. Biol. Chem. 239*, 486.
Breslow, E., Beychok, S., Hardman, K. D., & Gurd, F. R. N. (1965) *J. Biol. Chem. 240*, 304.
Cacece, M. S., & Cacheris, W. P. (1984) *Byte 9*, 340.
Cho, K. C., Poon, H. T., & Choy, C. L. (1982) *Biochim. Biophys. Acta 701*, 206.
Crumpton, M. J., & Polson, A. (1965) *J. Mol. Biol. 11*, 722.
Dautrevaux, M., Boulanger, Y., Han, K., & Biserte, G. (1960) *Eur. J. Biochem. 11*, 267.
deDuve, C. (1948) *Acta Chem. Scand. 2*, 264.
Draper, N. R., & Smith, H. (1966) in *Applied Regression Analysis*, p 282, Wiley, New York.
Edge, V., Allewell, N. M., & Sturtevant, J. M. (1985) *Biochemistry 24*, 5899.
Evans, S. V., & Brayer, G. D. (1988) *J. Biol. Chem. 263*, 4263.
Flanagan, M. A., Garcia-Moreno E., B., Friend, S. H., Feldmann, R. J., Scouloudi, H., & Gurd, F. R. N. (1983) *Biochemistry 22*, 6027.
Goldberg, A., & Dice, J. (1974) *Annu. Rev. Biochem. 43*, 835.
Goldberg, A., & St. John, A. C. (1976) *Annu. Rev. Biochem. 46*, 747.
Griko, Y. V., Privalov, P. L., Venyaminov, S. Y., & Kutysenko, V. P. (1988) *J. Mol. Biol. 202*, 127.
Guttman, I., & Meeter, D. A. (1965) *Technometrics 7*, 723.
Hanania, G. I. H., Yeghiayan, A., & Cameron, B. F. (1966) *Biochem. J. 98*, 189.
Hapner, K. D., Bradshaw, R. A., Hartzell, C. R., & Gurd, F. R. N. (1968) *J. Biol. Chem. 243*, 683.
Harrison, S. C., & Blout, E. R. (1965) *J. Biol. Chem. 240*, 299.
Herskovits, T. T., & Polson, A. (1965) *J. Mol. Biol. 11*, 722.
Herskovits, T. T., & Solli, N. J. (1975) *Biopolymers 14*, 319.

Holladay, L. A. (1985) *Biophys. Chem. 22*, 281.
Holladay, L. A. (1986) *Comp. Biochem. Physiol. 83B*, 365.
Kelly, L., & Holladay, L. A. (1987) *Biophys. Chem. 27*, 77.
Kelly, L., & Holladay, L. A. (1990) *Int. J. Pept. Protein Res. 35*, 235.
Kelly, L., Simmons, J. H., Heck, T., & Holladay, L. A. (1988) *Int. J. Pept. Protein Res. 31*, 281.
Luntz, T. L., Schejter, A., Garber, E. A. E., & Margoliash, E. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3524.
McLendon, G. (1977) *Biochem. Biophys. Res. Commun. 77*, 959.
Nelder, J. A., & Mead, R. (1965) *Computer J. 7*, 308.
Pace, C. N., & Vanderburg, K. E. (1979) *Biochemistry 18*, 288.
Pfeil, W. (1981) *Mol. Cell. Biochem. 40*, 3.
Privalov, P. L. (1979) *Adv. Protein Chem. 33*, 167.
Privalov, P. L., & Khechinashvili, N. N. (1974) *J. Mol. Biol. 86*, 665.
Privalov, P. L., & Gill, S. J. (1989) *Adv. Protein Chem. 39*, 191.
Privalov, P. L., Khechinashvili, N. N., & Atanasov, B. P. (1971) *Biopolymers 10*, 1865.
Privalov, P. L., Griko, Y. V., & Venyaminov, S. Y. (1986) *J. Mol. Biol. 190*, 487.
Puett, D. (1972) *Biochemistry 11*, 1980.
Puett, D. (1973) *J. Biol. Chem. 248*, 4623.
Puett, D., Friebele, E., & Hammonds, R. G., Jr. (1973) *Biochim. Biophys. Acta 328*, 261.
Romero-Herrera, A. E., & Lehmann, H. (1975) *Biochim. Biophys. Acta 400*, 387.
Romero-Herrera, A. E., Lieska, N., Friday, A. E., & Joysey, K. A. (1982) *Philos. Trans. R. Soc. London B297*, 1.
Sandberg, W. S., & Terwilliger, T. C. (1989) *Science 245*, 54.
Santoro, M. M., & Bolen, D. W. (1988) *Biochemistry 27*, 8063.
Schellman, J. A. (1978) *Biopolymers 17*, 1305.
Schellman, J. A. (1987) *Biopolymers 26*, 549.
Schellman, J. A., & Hawkes, R. B. (1980) in *Protein Folding* (Jaenicke, R., Ed.) p 331, Elsevier, New York.
Shortle, D., Meeker, A. K., & Gerring, S. L. (1989) *Arch. Biochem. Biophys. 272*, 103.
Takano, T. (1977) *J. Mol. Biol. 110*, 537.
Tanford, C. (1964) *J. Am. Chem. Soc. 86*, 2050.

Multiple Forms of $G_{o\alpha}$ mRNA: Analysis of the 3′-Untranslated Regions[†,‡]

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ABSTRACT: G_o, a guanine nucleotide binding protein found predominantly in neural tissues, interacts in vitro with rhodopsin, muscarinic, and other receptors and has been implicated in the regulation of ion channels. Despite the virtual identity of reported cDNA sequences for the α subunit of G_o ($G_{o\alpha}$), multiple molecular weight forms of mRNA have been identified in tissues from all species examined. To investigate the molecular basis for the size heterogeneity of $G_{o\alpha}$ mRNAs, four cDNA clones were isolated from the same retinal λgt10 cDNA library that was used earlier to isolate λGO9, a clone encompassing the complete coding region of $G_{o\alpha}$. These clones were identified as $G_{o\alpha}$ clones based on nucleotide sequence identity with λGO9 in the coding region; they diverge, however, from λGO9 in the 3′-untranslated region 28 nucleotides past the stop codon. An oligonucleotide probe complementary to a portion of the 3′-untranslated region of λGO9 that differs from the newly isolated clones hybridized with 3.0- and 4.0-kb mRNAs present in bovine brain and retina whereas a similar probe for the unique region of the new clones hybridized with a 4.0-kb mRNA in both tissues and with a 2.0-kb mRNA found predominantly in retina. A similar hybridization pattern was observed when brain poly(A+) RNA from other species was hybridized with the different 3′-untranslated region probes. It appears that differences in the 3′-untranslated regions could, in part, be the basis for the observed heterogeneity in $G_{o\alpha}$ mRNAs.

Structurally related guanine nucleotide binding proteins (G proteins)[1] couple many membrane-bound receptors to their intracellular effector proteins. This family includes the transducins (G_t), which link photoexcited rhodopsin in the retina to the cyclic GMP phosphodiesterase, G_s and G_i, which are responsible for stimulation and inhibition, respectively, of adenylyl cyclase, and G_o, a protein originally isolated from bovine brain (Sternweis & Robishaw, 1984; Florio & Sternweis, 1985; Stryer, 1986; Gilman, 1987; Moss & Vaughan, 1988). G_o interacts in vitro with rhodopsin, muscarinic, and other receptors (Kurose et al., 1986; Tsai et al., 1987) and has been implicated in ion channel regulation (Hescheler et al., 1987).

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[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02900.

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[1] Abbreviations: G protein, guanine nucleotide binding protein; G_t, retinal G protein transducin; $G_{t\alpha}$, α subunit of G_t; $G_{t\alpha 1}$, $G_{t\alpha}$ found in retinal rod outer segments; G_s and G_i, stimulatory and inhibitory G proteins coupled to adenylyl cyclase, respectively; $G_{s\alpha}$, α subunit of G_s; $G_{i\alpha}$, α subunit of G_i; G_o, G protein that may regulate ion flux; $G_{o\alpha}$, α subunit of G_o; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction.