A Correlation between Myoglobin Thermodynamic Stabilities and Species Metabolic Rates

George McLendon
Department of Chemistry
University of Rochester
Rochester, N.Y. 14627

Received June 6,1977

Summary

The free energy for total protein unfolding ($\Delta G_{N\rightarrow D}$) has been determined for myoglobins from whale, horse, cow and dog. A strong correlation between the $\Delta G_{N\rightarrow D}$'s and the basal metabolism rates of these species has been found. This correlation is shown not to reflect differences in protein oxygen affinity. In vitro proteolytic susceptibility studies show the relative susceptibilities vary inversely with $\Delta G_{N\rightarrow D}$. This and other considerations suggest that the correlation may reflect intrinsic (sequence determined) differences in turnover rates of homologous proteins.

Introduction

The factors which determine the stability of the tertiary structures of globular proteins are not well understood [1,2]. One experimental approach to this problem is to determine the free energy for protein unfolding [3-6]. Information on the roles of specific interactions in stabilizing a given tertiary structure may in principle be obtained by studying homologous proteins, in which one or more amino acid substitutions occur. Thus, Pace has studied the guanidine induced reversible denaturation of several β lactoglobulins [7] and cytochrome C's [8]. Puett has similarly examined the conformational stability of several myoglobins [9,10], ribonuclease derivatives [11] and growh hormones [1]]. It is clear from these results that differences in conformational stability exist among homologous proteins from different species. Unfortunately, it has generally proven unfeasible to uniquely interpret these differences [8,10]. Of equal importance, it is unclear whether these observed stability differences represent an effectively "neutral" mutation [13], or whether there is some specific under-

Copyright © 1977 by Academic Press, Inc.
All rights of reproduction in any form reserved.

ISSN 0006-291X

lying physiological basis for such differences. Puett has attempted to correlate myoglobin stability differences with evolutionary distance without success [10]. The observed range of cytochrome C stabilities led Pace to speculate that differences in protein conformational stability might be involved in control of protein catabolism [6,8]. This possibility has been discussed in somewhat more detail by Goldberg [14,15].

In an attempt to shed some light on some of these questions, thorough studies of the conformational stabilities of several groups of homologous proteins have been undertaken [16-19]. In the course of this work, we have discovered a remarkable correlation between the conformational stabilities of myoglobins from several species and the basal metabolism rates of those species. The implications of such a correlation are discussed below.

Materials and Methods

2X crystallised myoglobins from whale, horse, and dog were obtained from Sigma Chemicals, and further purified by column chromatography. Bovine myoglobin was prepared by the literature procedure [20] using fresh heart tissue obtained locally. All preparations had $\varepsilon 410 \geq 1.4 \times 10^5$ and showed only one major band on electrophonesis (A faint second band could sometimes be noticed.) "Ultra pure" guanidine hydrochloride was obtained from Schwarz Mann. Guanidine solutions were prepared by weight, and concentration checked by density. Titrations were performed batchwise. Temperature was maintained at 25.0°C using circulating cells. Denaturation was monitored by C.D. (JASCO J-40), visible spectra (Cary 14 or Gifford) or fluorescence (Perkin Elmer MPF 2). A minimum of 8 solutions within the transition region were used, and all determinations were performed in triplicate, so that a minimum of 24 points were available for data analysis. Data were analyzed by the standard methods, as reviewed by Pace [6]. All other methods for denaturation equilibrium determination are presented in detail elsewhere [16].

Proteolytic enzymes (trypsin, chymotrypsin, subtilism, and protease type V (a nonspecific protease from streptomyces griseus) were obtained in the highest available purity from Sigma Chemicals. Solutions of whale, horse, and dog myoglobins were incubated at 25.0°C with varying concentrations of the individual proteases. Substrate degradation was monitored by absorbance at 410 nm or CD at 220 nm.

Table 1. Conformational Free Energy of Myoglobins and Metabolic Data for Several Species.

Species	$\Delta G_{N\rightarrow D}$[a] kcal/M		Basal Metabolism Rate[d] kcal/kg/day
	[b]	[c]	
sperm whale	13.2	10.6	3.7[e]
cow	11.2	8.4	12
horse	10.6	7.6	17
human	8.8		25
dog		6.3	32

[a] $\Delta G_{N\rightarrow D}$ ≡ Free energy for transition from native to a randomly coiled 'totally' denatured state in the absence of denaturant. 25.0°, pH 7.1.

[b] Data of Puett ref. 10.

[c] This work, denaturant binding model.[7,8] An uncertainty of ~10% should be assumed.

[d] Reference 9.

[e] Calculated from the regression equation relating weight and basal metabolism rate,[9] assuming a weight of ~300,00 kg.

Results and Discussion

Results of denaturation studies of myoglobins from various species are summarized in Table 1. Both data from this work and the literature [10] are included. Acid denaturation data are not included due to apparent differences in the denatured state [16]. For the guanidine denaturations, extremely close correspondence to "two state" behaviour was found for all derivatives. High cooperativity (m > 4.5 kcal/M/M) was observed for the unfolding. This m value is greater than that previously reported [9,10], indicating the fit of the present data to a "two state" approxi-

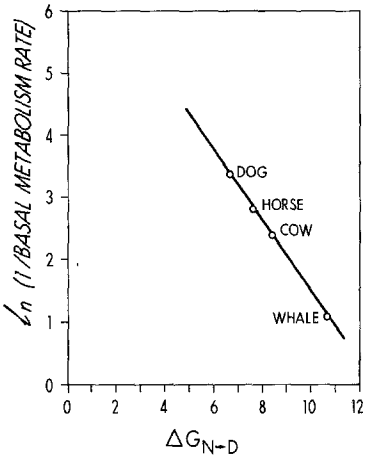

Figure 1

mation should be excellent. CD, fluorescence, and viscosity measurements indicated the denatured states are essentially identical. Observed stability differences thus probably reside in the native state. Detailed presentation and discussion of data are presented elsewhere [16]. Basal metabolism data are taken from the classic compilation of Brody [21]. As shown in figure 1, a strong correlation exists between the ln basal metabolism rate of a given species and the stability of myoglobin from that species. This is the first known correlation between protein stability and an important biological parameter. (As noted above, attempts to relate myoglobin stabilities to evolutionary distance have proven unsuccessful.) While interesting, the immediate interpretation of this correlation is unclear. (A student 't' test gives $p < .001$, obviating the possibility that the correlation is coincidental.) One suggested explanation [22] is that the observed correlation is related in some way to different requirements for oxygen supply among the species. Such a situation appears to exist in hemoglobins, where $P_{1/2}O_2$ is proportional to metabolic rate, apparently because of differing Bohr effects [23]. Two observations negate

this possibility for myoglobins. First, whereas the conformational stabilities determined herein vary by over 40%, the known myoglobin oxygenation free energies are constant within 2% for all species examined [20]. Selection for myoglobins of a different conformational stability is not accompanied by a corresponding change in function. Second, experiments in this lab on ligand induced differences in conformational stability [16,18] have shown that the conformational free energy increment on ligation (with concomittant spin change) is constant and independent of the initial conformational free energy of the myoglobin, for all species reported presently. Thus, the conformational stability expressed as $\Delta G_{N\rightarrow D}$ of a given myoglobin (and its relationship to metabolic rate) is not related to differences in oxygen demand of the various species. A quite different potential explanation follows. As metabollic rate increases, protein turnover also increases. For example, the physiological half lives of serum proteins from various species are strongly correlated with basal metabolic rates [24].*

It is generally accepted that protein turnover is usually regulated by proteolytic digestion of the substrate protein [14, 15]. Furthermore, proteins in the denatured state are much more susceptible to proteolysis [25,26,17]. The conformational free energy ($\Delta G_{N\rightarrow D}$) controls the equilibrium concentrations of native and unfolded (proteolytically susceptible) conformations. Thus, the possibility that the turnover rate of some proteins, including myoglobins, might be under "thermodynamic control" should be seri-

* The correlation implies a degradation mechanism. It is assumed that basal metabolism rate (BMR) is proportional to protein half life, as demonstrated for several serum proteins [24]. Since $t_{1/2} = .693/k$, $k \propto 1/B.M.R.$ Furthermore k (the rate constant for proteolysis) is proportional to K_D (the denaturation equilibrium constant) [17]. Thus $1/BMR \propto K_D$. In K_D is of course proportional to ΔG, thus $(\ln 1/BMR) \propto \Delta G$ if unfolding controls the half life of myoglobin in vivo.

ously entertained [27]. One consequence of such a hypothesis (in its simplest form) is that the rates of proteolytic digestion of myoglobins should vary inversely with their stabilities. (A strong correlation between in vitro proteolytic susceptibility and in vivo turnover rates has been established [14].) We have therefore examined the relative proteolytic susceptibilities of myoglobins from whale, horse, and dog to a variety of proteases (see Materials and Methods). Under "native state" conditions 25.0°, pH 7.0, so that the digestion rate should depend on K_D [27], the relative rates of digestion are whale < horse < dog, as predicted from their thermodynamic stabilities (Complete experimental details of these experimen will be presented elsewhere [17].)

It must be noted, of course, that in vivo turnover data are not available for myoglobins; nor are they likely to be for such species as whale. Thus the present results do not prove that myoglobin turnover is thermodynamically controlled. Nevertheless, the interesting correlation between metabolic rate and stability, coupled with the proteolytic data suggest that this possibility should be strongly considered. The critical test of this hypothesis must await determination of conformational free energies for proteins of known in vivo half life. Such studies are proceeding in this laboratory.

Finally, it is interesting to compare the limited data available for other protein types. As noted above, Pace first broached the possibility of thermodynamic turnover regulation on the basis of differences in the stabilities of homologous cytochrome c's [8]. We have since carried out extensive studies of cytochrome c's from eight species,and find no correlation between the metabolic rate of a given species and the stability of cytochrome c from that species [19,28]. It should be noted in this

context that the regulation of mitochondrial protein turnover is not simple. Varying parts of the mitochondrion appear to turn over as discrete units, with little or no heterogeneity in the degradation rates of individual component proteins [29].

References

1. C. Anfinsen and H. Scheraga. Adv. Pro. Chem. 28, 205 (1975).
2. D. Weltaufer and S. Ristow. Ann. Rev. Biochem. 43, 419 (1974).
3. C. Tanford, Adv. Prot. Chem. 23, 121 (1968).
4. C. Tanford, ibid., 24, 1 (1970).
5. H. Edelhoch and J. Osborne, ibid., 29, 183 (1976).
6. C. N. Pace. Crit. Revs. Biochem. 3, 1 (1975).
7. S. Alexander and C. N. Pace. Biochem. 10, 2738 (1971).
8. J. Knapp and C. N. Pace. Biochem. 13, 1289 (1974).
9. D. Puett, J. Biol. Chem. 248, 4623 (1973).
10. D. Puett, E. Frieble, R. Hammonds. Biochem. Biophys. Acta 328, 261 (1973).
11. D. Puett. Biochem. 11, 1980 (1972).
12. L. Holladay, R. Hammonds, D. Puett. Biochem. 13, 1653 (1974).
13. J. King and T. H. Jukes. Science 158, 1417 (1967).
14. A. Goldberg and J. Dice. Ann. Revs. Biochem. 44, 835 (1974).
15. A. Goldberg and A. C. St. John. Ibid., 96, 747 (1976).
16. G. McLendon and K. Sandberg. Biochem., submitted.
17. G. McLendon and E. Haftkowycz, to be published.
18. G. McLendon. Biochem. Biophys. Acta., submitted
19. G. McLendon and M. Smith. J. Biol. Chem., submitted.
20. E. Antonini and M. Brunori. Hemoglobin and Myoglobin in Their Reactions with Ligands, Elsevier, N.Y. 1971.
21. S. Brody. Bioenergetics and Growth, Reinhold, N.Y. (1945).
22. Alfred Goldberg, personal communication.
23. S. Tomita and A. Riggs. J. Biol. Chem. 246, 547 (1971).
24. S. Munro. Mammalian Protein Metabolism, Elsevier, N.Y. 1968.

25. J. Rupley and H. Scheraga. Biochem. 2, 421 (1963).

26. J. Bond. Biochem. Biophys. Res. Comm. 43, 333 (1971).

27. The simplest mathematical expression would be
$$-\frac{d\ protein}{dt} = kK_D[Protein]_N[Proteolytic\ Enzyme]$$
where k is the rate constant for digestion of the unfolded form and K_D the equilibrium constant for unfolding.

28. This is equally obvious by sequence inspection. Dog and Panda have widely different metabolic rates, but the same sequence, and thus the same stability. The same is true of cow and sheep. (E. Margoliash, personal communication).

29. M. Rabinowitz. Am. J. Cardiology 31, 202 (1973).