Conformational stability and basal metabolic rate: reexamination of the case of myoglobin

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Summary. The free energy of unfolding of several myoglobins from different animal species has been determined from their denaturation pattern by using the ligand binding model. The results indicate that no simple correlation exists between the free energy of unfolding of myoglobin and the basal metabolic rate of the animal species from which the myoglobin was isolated.
Key words. Myoglobin; conformational stability; basal metabolic rate; unfolding, free energy of.

Attempts to explain in vitro differences in protein metabolic rate on the basis of physical properties such as the conformational stability have led to contrasting conclusions[1,2]. In fact, recently evidence was given by McLendon[3] for a linear relationship between the in vitro conformational stability of four mammalian myoglobins and the basal metabolic rates of the corresponding animal species. However, the same authors also reached opposite conclusions when studying the susceptibility to denaturation of homologous cytochrome c's[4].

The results reported in this paper, obtained by widening the number of animal species, have shown that the free energy of unfolding of myoglobins appears not to be linearly related to the corresponding metabolic rates, thus supporting McLendon's results obtained on cyt c[4].

Materials and methods. Myoglobins were prepared according to the methods previously described[5,6], except that horse and sperm whale myoglobins which were purchased from Sigma. All proteins were used after a run on Sephadex G-75 (2.5 × 100 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0.

The denaturation equilibria of myoglobins were measured at 25 °C following the dichroic activity at 222 nm. Circular dichroism (CD) measurements were carried out on a Jobin Yvon MK3 spectropolarimeter, equipped with a temperature controlled cell holder.

In denaturation experiments, the protein was added to solutions of guanidine hydrochloride (Schwartz/Mann) (GuHCl) buffered with 0.05 M phosphate buffer, pH 7.0; 0.1 M KCl was present in all solutions.

Results and discussion. The free energy of unfolding of a large number of myoglobins has been calculated from their denaturation pattern at neutral pH and at 25 °C. GuHCl was used as a denaturant because of its ability to produce randomly coiled polypeptides. CD spectra in the far UV and intrinsic viscosities of denatured myoglobins indicated that the unfolded state provides in all cases a common reference state. No peptide structure was detected by CD in the far ultraviolet region (205–240 nm) in 4.0 M GuHCl, pH 7.0; in the same solvent intrinsic viscosities ranged between 20–22 ml/g for the different myoglobins in good agreement with the theoretical value predicted for a randomly coiled protein of 153 amino acid residues[7].

Free energy of unfolding of myoglobins

Species	a	b	c	d	e
Pig	11.3 ± 0.4	–	–	–	–
Buffalo	10.0 ± 0.2	–	–	10.5	–
Cow	10.7 ± 0.4	11.2	–	11.5	8.4
Human	8.3 ± 0.3	8.8	–	–	–
Horse	9.9 ± 0.3	10.6	10.1	–	7.6
Tuna 25 °C	9.2 ± 0.3	–	–	9.1	–
Sperm whale	12.5 ± 0.4	13.2	–	–	10.6
Dog	8.9 ± 0.2	–	–	–	6.3
Turtle	8.0 ± 0.4	7.2[f]	–	–	–
Aplysia	8[g]	–	–	–	–

[a]Data reported in this paper (25 °C). [b]From references 11 and 12. [c]From reference 10. [d]From reference 6. [e]From reference 3. [f]Determined at 5 °C. [g]Calculated from reference 16.

The GuHCl unfolding has been studied following the effect of increasing denaturant concentration on the α-helix content of the proteins. The transition curves obtained by plotting the spectral changes at 222 nm vs. GuHCl concentration were steep and sigmoidal and were treated as a simple equilibrium between two states, i.e. native (n) and denatured (d).

The free energy of unfolding at each concentration of denaturant was calculated by using the equation:

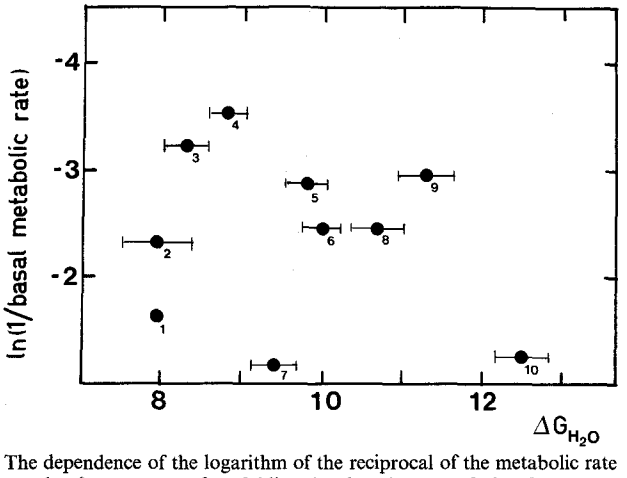
$$\Delta G_D = -RT\ln((x_n - x_i)/(x_i - x_d)) \quad (1)$$

where x_i is the numerical value of the structure sensitive parameter at the ith denaturant concentration; x_n and x_d are the numerical values of the same parameter relative to the native and fully denatured state, respectively.

The free energy of unfolding in the absence of denaturant ($\Delta G_D^{H_2O}$) has been obtained by using the ligand binding model[8,9]. This model assumes that the denaturation is due to the binding of the denaturant to the protein. This assumption leads to the following equation:

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT\ln(1 + ka_{\pm}) \quad (2)$$

where k is the average binding constant, $a_{\pm}$ the mean ionic activity of the denaturant, and n the difference between the number of denaturant binding sites in the denatured state and the number of binding sites in the native state. According to the suggestion recently made by Pace and Vanderburg[10] we have used a value of 0.6 for k in our computations. For each myoglobin examined the ΔG_D values calculated from equation (1) at several denaturant concentrations were fitted to equation (2) in order to obtain the $\Delta G_D^{H_2O}$ as well as Δn.



The dependence of the logarithm of the reciprocal of the metabolic rate on the free energy of unfolding in the absence of the denaturant ($\Delta G_D^{H_2O}$). The metabolic rates were taken from Brody[13]. The numbers refer to the species: 1, *Aplysia;* 2, turtle; 3, man; 4, dog; 5, horse; 6, buffalo; 7, tuna; 8, cow; 9, pig; 10, sperm whale. The bars indicate the error associated to the calculation of $\Delta G_D^{H_2O}$ except for *Aplysia*, the $\Delta G_D^{H_2O}$ of which was taken from reference 16. Each value represents an average on five measurements.

The table shows the results obtained on nine myoglobins in comparison with the data available in the literature and obtained by using the same computation method. Consistent similarities exist among the different sets of data except for the data taken from McLendon[3] which are considerably lower than those presented in this paper and those available in the literature. The small discrepancy between our $\Delta G_{D^2O}^{H_2O}$ values and those taken from Puett[11,12] is due to the numerical value of k (0.6 in our computation, 1.2 in Puett's computation).

The figure shows a plot of the logarithm of the reciprocal of the metabolic rate vs the free energy of unfolding of the myoglobins examined. This kind of plot has already been used to correlate metabolic rates and susceptibilities to unfold of different protein classes[3,4]. However we tried to plot the data in several different ways in order to have a correlation but the results were meaningless. Basal metabolism data were taken from the classic compilation of Brody[13]. No linear correlation appears to exist between the two sets of data; in fact, the least square analysis of the data gave a very poor correlation factor[14], i.e. $r^2 = 0.14$. Therefore, it appears evident that the linear correlation found by McLendon is accidentally limited to the four myoglobins examined by the same author, i.e. dog, horse, cow and whale myoglobins. Widening the number of animal species leads to the conclusion that no simple correlation between the free energy of unfolding and the metabolic rate exists. It is worth taking into consideration that $\Delta G_{D^2O}^{H_2O}$ is a temperature-dependent function; therefore one might expect that the correlation shown in the figure would be improved if $\Delta G_{D^2O}^{H_2O}$ determinations were made at physiological temperatures rather than 25 °C. Among the proteins considered in this paper, seven were from warm-blooded animals with similar physiological temperatures, and the other three from poikilotherm species. Privalov and Khechinashvili[15] reported recently that the free energy of unfolding of myoglobins is fairly constant between 20 and 40 °C and drops at temperatures lower than 20 °C as well as higher than 40 °C. In this respect, the correlation betwen the two sets of data reported in the figure would be further weakened. In fact, while the $\Delta G\Delta G_{D^2O}^{H_2O}$ of myoglobins of warm-blooded animal species is expected to be quite similar to that obtained at 25 °C, a fall in temperature from 25 to 5 °C results in a decrease of $\Delta G_{D^2O}^{H_2O}$ for poikilotherm myoglobins as shown by Puett in the case of turtle myoglobin (table). Moreover, it must be pointed out that these results extend similar observations made previously on homologous cytochrome c's[15].

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5′-Methyl-cytosine in the macronuclear DNA of *Blepharisma japonicum*

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Summary. Brief report on the presence of 5′-methyl-cytosine as a minor base (0.56%) in the macronuclear DNA of the ciliate protozoan *Blepharisma japonicum*. The evidence comes from electrophoresis of macronuclear DNA digested by appropriate restriction endonucleases and high-performance liquid chromatography.
Key words. Protozoa, ciliate; ciliates; *Blepharisma japonicum;* DNA, macronuclear; 5′-methylcytosine.

Methylated adenine (MeAde) and methylated cytosine (MeCyt) in nuclear DNA occur widely in a great variety of organisms from prokaryotes to plants and animals[1].

This base methylation of nuclear DNA has mainly been considered for its relevance in DNA information transfer and much less from the evolutionary point of view. There is good evidence for a relationship of base methylation with the restriction/modification systems in prokaryotes[1] and with other phenomena of DNA function as well[2]. Restriction/modification systems have not been clearly demonstrated in eukaryotes[3], but other functions have been postulated for base methylation, such as the involvement of cytosine methylation in spontaneous mutagenesis, in the determination of the higher order of chromosome structure, and particularly in the control of gene transcription during differentiation[4].

From the evolutionary point of view it is interesting to note that methylation of both adenine and cytosine may occur in prokaryotes[5], though in some species only one base becomes methylated. In contrast, MeCyt is the only methylated base yet found in multicellular eukaryotes. The only exceptions we know of are cultivated cells of *Aedes albopictus,* and salmon sperm, in which a small amount of MeAde has been found[6,7]. The situation in unicellular eukaryotes (protists) is more variable than in multicellular eukaryotes. In some phytoflagellates both MeAde and MeCyt were found, and in dinoflagellates 5-hydroxymethyl-uracil is predominantly present together with