TEMPERATURE-INDUCED CONFORMATIONAL CHANGES IN SPIN-LABELLED MYOGLOBINS: MYOGLOBIN OF *APPLYSIA BRASILIANA*

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1. Introduction

To obtain a detailed understanding of both the general structure and function of specific members of the class of hemoproteins, comparative studies using homologous proteins from a wide variety of organisms are very useful. Myoglobins from a number of invertebrate species have been characterized both structurally and functionally [1]. Myoglobin of *Applysia limacina* was isolated, characterized [2,3] and several structural and functional properties have been studied [4–7]. *Applysia brasiliana* is common on the eastern coast of Brazil.

Here, we report results on temperature-induced changes of spin-labelled myoglobin of *Applysia brasiliana* and compare the results with sperm-whale myoglobin, the denaturation properties of which are interesting [2,4,5,8].

2. Material and methods

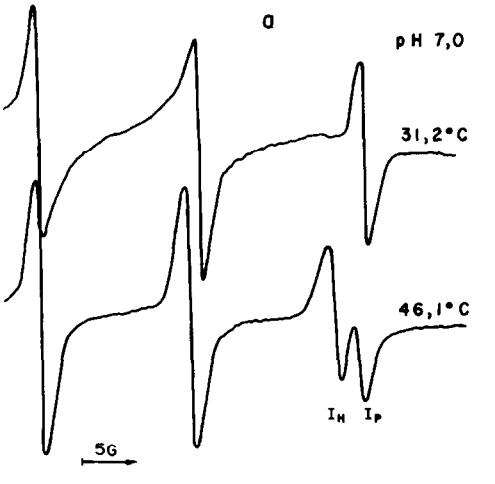
Applysia brasiliana were collected in San Sebastião (São Paulo) during summer and the radular muscle isolated [9,10]. Myoglobin was purified by precipitation with ammonium sulfate [2] and column chromatography in DEAE-cellulose [11]. Two main fractions were obtained and the more intense one used, as in denaturation [9] and acid–alkaline transition studies [11]. The myoglobin used was always in the Met form.

After elution from the column the myoglobin was concentrated to ~1–2% and reacted with isothyocyanate spin label (103, from Syva Co.). The reaction was made by incubation of the label with myoglobin solution at 4°C with mixing for ~72 h. The label/myoglobin ratio was 2:1 M. After the reaction the myoglobin was dialysed against the appropriate buffer to remove free spin label. The buffers used were Tris 0.05 M with adjusted pH values.

EPR spectra were taken with a Varian X band E-9 spectrometer equipped with a system for temperature control.

3. Results

In fig.1 the EPR spectra of spin-labelled myoglobin of *A. brasiliana* are shown at 2 different temperatures and for different pH values. As the temperature increases a splitting at the high field component ($M_I = -1$) occurs which is indicative of a conformational change in the microenvironment of the label. With further increase in temperature the signal due to the label in the more hydrophobic environment I_H increases and the signal due to the label in the polar environment I_P decreases. This conformational



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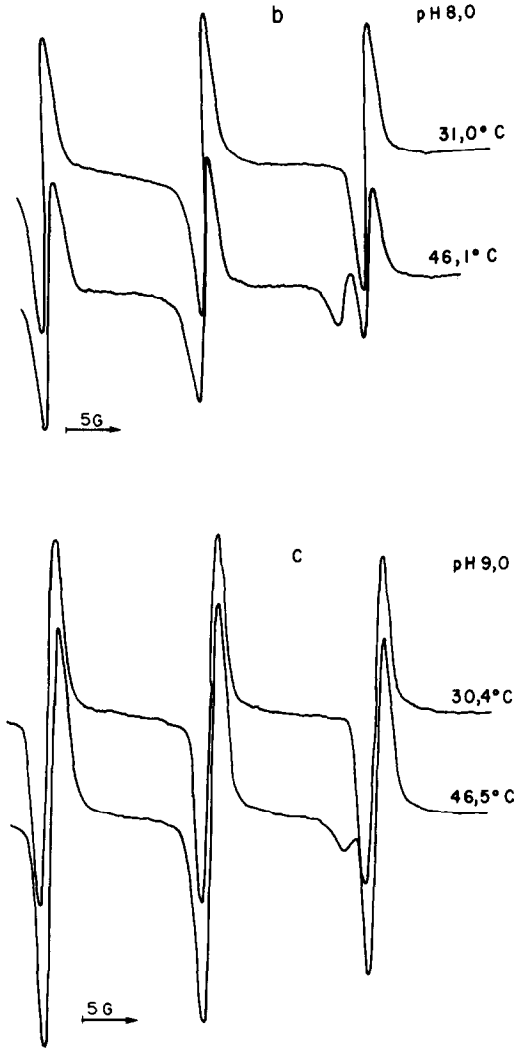

Fig.1.(a) EPR spectra of spin-labelled myoglobin of *Applysia brasiliana* at 31.2°C and 46.1°C (pH 7.0); (b) EPR spectra of spin-labelled myoglobin of *Applysia brasiliana* at 31.0°C and 46.1°C (pH 8.0); (c) EPR spectra of spin-labelled myoglobin of *Applysia brasiliana* at 30.4°C and 46.5°C (pH 9.0).

change is very sensitive to the pH value and is more evident at the low pH (pH 7.0). In table 1 values of EPR parameters calculated for 25°C are given as a function of pH. This temperature was chosen because no splitting of the high field line is observed. The EPR parameters calculated were the parameters B and C, coefficients in the expression for the linewidth:

$$\Delta H(M) = A + BM + CM^2$$

with

$$A = \Delta H(0)$$

$$B = \frac{\Delta H(0)}{2}\left(\sqrt{\frac{I(0)}{I(1)}} - \sqrt{\frac{I(0)}{I(-1)}}\right)$$

and

$$C = \frac{\Delta H(0)}{2}\left(\sqrt{\frac{I(0)}{I(1)}} + \sqrt{\frac{I(0)}{I(-1)}} - 2\right)$$

where $\Delta H(M)$ is the linewidth of the hyperfine components ($M = +1, 0, -1$) and $I(M)$ is the corresponding intensity of the lines. The values of τ_B and τ_C, the rotational correlation times corresponding to B and C were calculated as in [12] and also τ, the rotational correlation time was calculated from the expression:

$$\tau = 6.5 \times 10^{-10} \times \Delta H(1)\left(\sqrt{\frac{I(1)}{I(-1)}} - 1\right)$$

where $\Delta H(1)$ and $I(1), I(-1)$ have the same meaning as above. At pH 7.0 even at this temperature the spectrum was distorted and so the values of B and C were not calculated.

Table 1
EPR parameters as a function of pH

pH	B	C	τ_B (s)	τ_C (s)	τ (s)
7.0	–	–	–	–	2.3×10^{-10}
8.0	−0.057	0.090	0.7×10^{-10}	1.1×10^{-10}	0.35×10^{-10}
9.0	−0.025	0.086	0.3×10^{-10}	1.0×10^{-10}	0.29×10^{-10}

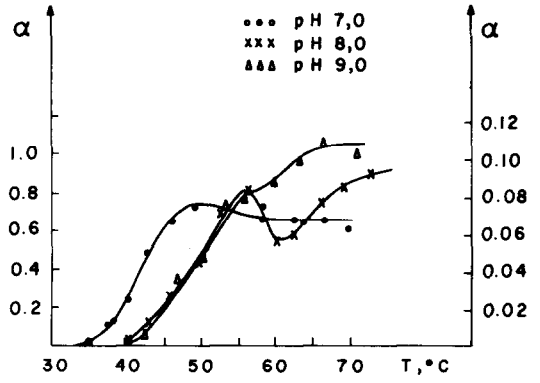

Fig.2. Temperature variation of the parameter $\alpha = I_H/(I_H + I_p)$ for spin-labelled myoglobin of *Applysia brasiliana* (pH 7.0; pH 8.0; pH 9.0, right axis).

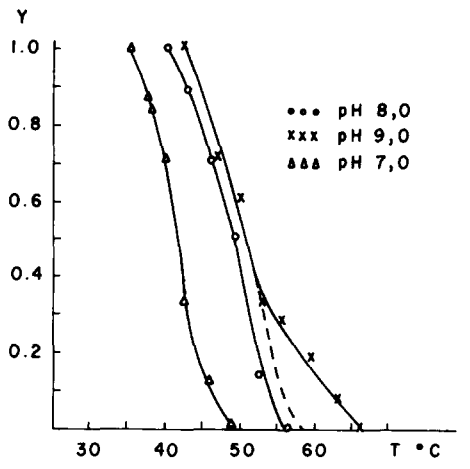

Fig.3. Temperature variation of the parameter $Y = (\alpha_{max} - \alpha/\alpha_{max} - \alpha_{min})$ for spin-labelled myoglobin of *Applysia brasiliana* (pH 7.0; pH 8.0; pH 9.0).

It can be seen that the values of τ, the rotational correlation time, decrease sharply with increase in pH. This means that in *Applysia* myoglobin the label is in a site sensitive to the acid–alkaline transition. While in the sperm whale myoglobin the pK of the acid–alkaline transition is ~9.0 in *Applysia* myoglobin this pK is ~7.0 [6,11]. In fig.2 we plotted the parameter:

$$\alpha = I_H/(I_H + I_p)$$

where I_H and I_p, respectively, the intensities of the components at the lower (hydrophobic) field and the higher (polar) field for the splitted line ($M_I = -1$). If the linewidths of the hydrophobic and polar lines are approximately equal this ratio is the ratio between the respective areas and so is proportional to the fraction of label in the hydrophobic environment. A strongly pH-dependent transition with temperature is seen to occur. Besides the 35–42°C range at which the hydrophobic component appears, there is also a less pronounced change near 50–55°C, seen most clearly at pH 8.0 (fig.2).

In fig.3 another plot is given in an attempt to obtain more precisely the transition temperatures for the conformational changes. The parameter used in that figure is:

$$Y = (\alpha_{max} - \alpha)/(\alpha_{max} - \alpha_{min})$$

where α_{max} was defined over 55–60°C and has values from 0–1. At lower pH the transition occurs at lower temperature (fig.3).

4. Discussion and conclusions

In table 2 we compare the amino acid residues of myoglobin of sperm whale and myoglobins of *A. limacina* [7] and *A. brasiliana* [11]. In *Applysia* myoglobin the only histidine residue is the proximal one. However, the number of aromatic residues is much higher

Table 2
Comparison of amino acid[a] residues in different myoglobins as percent of total content

Source	Acids (Asp + Glu)	Basics (Lys + Arg + His)	Aromatics (Phe + Tyr + Trp)	Hydrophobics (Leu + Val + Ile + Phe)
A. brasiliana[b]	18.4	14.2	17.2	28.8
Mollusc	9.7	11.7	11.7	29.0
Insect	9.6	12.5	12.5	30.9
Vertebrate	13.7	21.8	7.2	27.6

[a] From [16]; [b] from [11]

than in the sperm whale myoglobin. These facts are probably responsible for the lower pI value for myoglobin of *Applysia* as well as for the lower pK value of acid–alkaline transition. Since it has been shown that the NH_2-terminal of *Applysia* myoglobin is blocked [11] thus it is quite probable that some aromatic residue is modified upon reaction with isothiocyanate spin label. Modifying the area of the NH_2-terminal of sperm-whale myoglobin affects heme region [13,14]. If this is a general property of myoglobins our spin label may be in an aromatic residue in the region of the NH_2-terminal. The calculated values of τ are characteristic of a weakly immobilized label at the protein surface.

Denaturation studies in [8,9] were made at pH 9.0 giving a transition temperature of $T_C = 66°C$. At this pH we have only the alkaline form of metmyoglobin of *Applysia*. The T_C at this pH obtained from our results is 51°C, lower than that obtained for native myoglobin. It is possible that the label facilitates the denaturation of myoglobin. This effect was observed for myoglobin labelled with Cu^{2+} in [15]. Further changes occur at the highest temperatures (fig.3a). These changes could be related to the denaturation of the protein.

At lower pH values both the T_C decreases (41.7°C at pH 7.0) and the amount to hydrophobic spin label increases considerably. This is especially evident at pH 7.0 where the spectrum is distorted showing a superposition even at lower temperatures. As the acid–alkaline transition occurs at pH ~7, the temperature may be very effective in shifting this equilibrium. The hydrophobic component is characteristic of the acid myoglobin (high-spin) and the polar component of the alkaline (low-spin). At pH 7.0, both spin forms are present at equal concentration. As the pH increases, the acid form is reduced and so is the hydrophobic signal. The temperature-induced transition is very sensitive to the myoglobin spin form.

Besides its thermal denaturation properties, *Applysia* myoglobin may be a useful system to examine spin equilibrium in hemoproteins.

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References

[1] Antonini, E. and Brunori, M. (1971) Hemoglobin and myoglobin in their reaction with ligands, North-Holland, Amsterdam.
[2] Rossi-Fanelli, A. and Antonini, E. (1957) Biokhymia 22, 335–344.
[3] Rossi-Fanelli, A., Antonini, E. and Povolado, D. (1960) in: Symposium on Protein Structure (Neuberger, A. ed) pp. 143–147, Methuen, London.
[4] Brunori, M., Antonini, E., Fasella, P., Wyman, J. and Fanelli, A. R. (1968) J. Mol. Biol. 34, 497–504.
[5] Brunori, M., Giacometti, G. M., Antonini, E. and Wyman, J. (1972) J. Mol. Biol. 63, 139–152.
[6] Giuseppe, R., Calabrese, L., Giacometi, G. M. and Brunori, M. (1971) Biochim. Biophys. Acta 236, 234–237.
[7] Tentori, L., Vivaldi, G., Carta, S., Marinucci, M., Massa, A., Antonini, E. and Brunori, M. (1973) Int. J. Pept. Prot. Res. 5, 187–200.
[8] Giacometti, G. M., Antonini, E. and Brunori, M. (1979) Biophys. Chem. 10, 119–127.
[9] Sekino, T., Focesi, A. jr, Bonaventura, C. and Bonaventura, J. (1978) IRCS Med. Sci. Biochem. 6, 261.
[10] Sekino, T., Focesi, A. jr, Bonaventura, C. and Bonaventura, J. (1978) Comp. Biochem. Physiol. 61A, 223–226.
[11] Say, J. C. (1980) PhD Thesis, Ribeirão Preto, São Paulo.
[12] Polnaszek, C. F. (1982) Quart. Rev. Biophys. in press.
[13] Di Marchi, R. D., Neireiter, G. W., Heath, W. F. and Gurd, F. R. N. (1980) Biochemistry 19, 2454–2465.
[14] Artiyukh, R. I., Atanasov, B. P. and Volkenstein, M. V. (1977) Molekul. Biol. 11, 410–416.
[15] Wanderley Louro, S. (1974) MSc Thesis, PUC/RJ.
[16] Dayhoff, M. O. (1976) in: Atlas of Protein Sequence and Structure, vol. 5, suppl. 2, Natl. Biomed. Res. Found., Silver Spring MD.