Extreme thermostability of tarantula hemocyanin

Reinhard Sterner[a], Thomas Vogl[b], Hans-Jürgen Hinz[b], Franz Penz[c], Rainer Hoff[c], Roman Föll[c], Heinz Decker[c,*]

[a]*Dept. of Biophysical Chemistry, Biozentrum, University of Basel, Klingelbergstr. 70, 4056 Basel, Switzerland*
[b]*Institute of Physical Chemistry, University of Münster, Schloßplatz 4/7, 48149 Münster, Germany*
[c]*Zoological Institute, University of Munich, Luisenstr. 14, 80333 München, Germany*

Received 14 March 1995

Abstract Biotops with extreme temperatures such as deserts force animals to avoid or escape high temperatures by biochemical, behavioural or morphological adaptation. In this context we tested the resistance to heat of the oxygen carrier hemocyanin from the ancient tarantula *Eurypelma californicum*, which is found in arid zones of North America. Differential scanning calorimetry, light scattering, crossed immunogelelectrophoresis and oxygen binding experiments show that the 24-meric hemocyanin is conformationally stable and fully functioning at temperatures up to 90°C. Our results demonstrate that the cation-mediated state of oligomerization is not only crucial for the high cooperativity of oxygen binding of this hemocyanin, but also for its extreme stability in the physiological temperature and pH range.

Key words: Hemocyanin; Diffential scanning calorimetry; Light scattering; Oxygen binding

1. Introduction

The tarantula *Eurypelma californicum* is found in the deserts of southwest North America where it faces high absolute temperatures and large temperature fluctuations of more than 70°C between day and night. Under these extreme conditions the oxygen supply of the animal has to be maintained. This means that its respiratory protein, the hemocyanin, has to be highly adaptable to drastic environmental changes with respect to its functional properties such as cooperativity and allosteric regulation of oxygen binding. At the same time the protein must be stable over a wide temperature range.

Hemocyanin is freely dissolved in the blood of *Eurypelma californicum* in a concentration between 10–120 g/l [1]. Fig. 1 shows that it is composed of 24 subunits, which belong to seven different types (designated with lower case letters *a* to *g*; [2,3]). Electron microscopic pictures revealed the arrangement of the subunits in 4 hexamers [4]. Two hexamers, which differ by containing either subunit *b* or *c*, are forming dodecamers that dimerize isologously to the native 24-meric hemocyanin. Isolated subunits reversibly bind one molecule of oxygen between two copper atoms in a non-cooperative manner and with high affinity [5]. In contrast, the 24-mer shows a highly cooperative oxygen binding with Hill coefficients of up to 9 and a reduced oxygen affinity [6,7]. Whereas a great amount of information has been accumulated on the functional properties of tarantula and related arthropod hemocyanins (for reviews see [8–10]), only few data on the stabiliy of this important class of respiratory proteins are available [11]. Here, we report measurements showing that the native 24-meric tarantula hemocyanin remains intact up to 90°C and below pH values of 6.2. It thus fulfills the stability requirements of a respiratory protein in the blood of a poikilothermic animal living under extreme environmental conditions.

2. Material and methods

Tarantula (*Eurypelma californicum*) hemocyanin was prepared as described [12] and immediately dialysed against 100 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ at 20°C. Under these conditions, the hemocyanin is a pure 24-mer [7]. The protein concentration was determined spectroscopically with the absorption coefficient $E^{1\%}_{278} = 11.1$ in Tris-HCl buffer.

2.1. Spectroscopic methods

Absorption spectroscopy was performed with a CARY spectrophotometer (model 114) at the given temperatures.

2.2. Differential scanning calorimetry

Differential scanning calorimetry was performed with both the 24-meric hemocyanin and with the isolated subunits. A DASM 4 microcalorimeter with a cell volume of 0.47 ml was used. The experiments were performed under air pressure of approximately 3 bar. Several runs were made from 20°C to 120°C with each sample, and the transition temperatures reported are average quantities (± 0.5°C). A heating rate of 1 K · min^{-1} was employed. Three different buffer systems were used:
(1) 100 mM Tris-HCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 8.8 at 20°C with a protein concentration of 1.32 g/l (24-meric form).
(2) 50 mM glycine/OH, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 9.6 at 20°C with a protein concentration of 1.0 g/l (24-meric form).
(3) 50 mM glycine/OH, 5 mM EDTA, pH 9.6 at 20°C with a protein concentration of 0.92 g/l (monomeric form).

Unfolding was irreversible in each case as judged on the basis of zero excess heat capacity in the second heating.

2.3. Light-scattering

Light scattering measurements were performed with a SPEX Fluorolog 211 spectrophotometer at a wavelength of 400 nm. The hemocyanin (1.0 g/l in 100 mM Tris-HCl buffer, pH 7.6 at 20°C containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$) was heated up with a rate of 1 K/min. The concomittant change of the pH value was measured with a pH minielectrode (Eschweiler, Germany). After a desired temperature had been reached, an aliqot of the sample was taken and further incubated at this temperature for 10 min. Then the samples were cooled down to 20°C and filtered into the cuvette (d = 1 cm), using Millipore filters with a pore width of 0.2 mm.

2.4. Crossed immunogelelectrophoresis

Tarantula hemocyanin was exposed for 10 min to different temperatures and then cooled down to 20°C. Subsequently, crossed immunogelelectrophoresis was performed according to Weeke [13] as modified by Markl et al. [14], using polyclonal rabbit antibodies against dissociated *Eurypelma californicum* hemocyanin.

*Corresponding author. Institute of Molecular Biophysics, University of Mainz, Welder-Weg 26, 55099 Mainz, Germany. Fax: (49) (6131) 393557.

SSDI 0014-5793(95)00341-X

2.5. Oxygen binding

Aliquots of the hemocyanin solution that had been exposed to different temperatures, were cooled down to 20°C and diluted to a concentration of 0.1 g/l with 100 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. With this solution, continuous oxygen-equilibrium curves were recorded with the fluorimetric-polarographic method [6].

3. Results and discussion

Structure and function of tarantula hemocyanin were examined as a function of temperature in the range from 20°C to 120°C by differential scanning calorimetry (DSC), light scattering, immunogelelectrophoresis and oxygen binding. Conformational stability of the 24-meric hemocyanin was investigated in two different buffer systems and in presence of physiological concentrations of Ca^{2+} and Mg^{2+} ions.

In Tris-buffer with 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 6.8 at 92.0°C) a transition temperature at the maximum of the excess heat capacity peak of T_m = 92.5°C was observed (Fig. 2, curve A). In glycine-buffer with 5 mM $CaCl_2$ and 5 mM $MgCl_2$ (pH 7.8 at 90°C) the transition temperature T_m is 89.9°C (Fig. 2, curve B), which suggests that the hemocyanin has the same degree of oligomerization as in Tris-buffer. Above this temperature the protein aggregates irreversibly, which precludes determination of heat capacity changes. Irreversibility of denaturation was also observed in DSC measurements with the hemocyanin from the lobster *Panulirus vulgaris* [11]. However, the hexameric lobster hemocyanin is much less heat resistant than the 24-meric tarantula hemocyanin. It denatures already at 63°C when a comparable scan rate of 1 K · min^{-1} is applied. We also performed DSC studies on a mixture of isolated tarantula hemocyanin subunits, which are produced at high pH and in the absence of Ca^{2+} and Mg^{2+} ions in glycine buffer [15]. The excess heat capacity curve C is shown in Fig. 2 and reveals a transition temperature of T_m = 68.4°C. This result indicates a lower stability of the isolated subunits compared to the 24- mer, but an increased stability with respect to subunits from lobster hemocyanin, which denature at 49°C [11].

Based on the DSC experiments performed with hemocyanins so far, the extreme thermostability of tarantula hemocyanin is

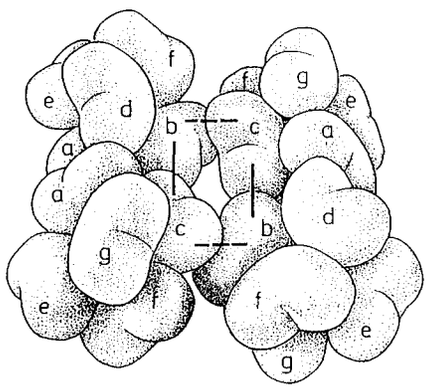

Fig. 1. Quaternary structure of the hemocyanin from the tarantula *Eurypelma californicum*. The hemocyanin is composed of 24 subunits, which belong to seven different types (designated with lower case letters *a* to *g* [2,9]. The subunits are arranged in 4 hexamers. Two hexamers, which contain either subunit *b* or *c*, are forming dodecamers that dimerize isologously to the native 24-meric hemocyanin (adapted from [13]).

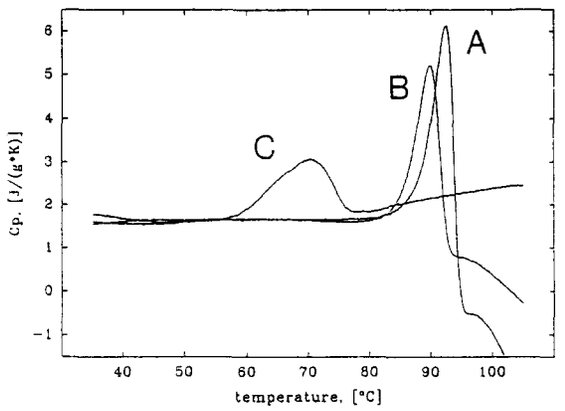

Fig. 2. Extreme thermostability of tarantula hemocyanin depends on its 24-meric oligomerization state. Differential scanning calorimetric studies were performed on 24-meric and subunits of hemocyanin. A heating rate of 1 K · min^{-1} was employed throughout the measurements. Curve A: 100 mM Tris-HCl, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 8.8 at 20°C; protein concentration: 1.32 g/l (24-meric form). Curve B: 50 mM glycine/OH, 5 mM $CaCl_2$ and 5 mM $MgCl_2$, pH 9.6 at 20°C; protein concentration: 1.0 g/l (24-meric form). Curve C: 50 mM glycine/OH, 5 mM EDTA, pH 9.6 at 20°C; protein concentration: 0.92 g/l (monomeric form).

due to its high state of oligomerization and to an elevated intrinsic stability of its subunits compared to lobster hemocyanin. Since the T_m values of the 24-mer in Tris and glycine buffer are almost identical (Fig. 2, curves A,B), the observed high thermostability of tarantula hemocyanin seems to be an intrinsic property which is only slightly influenced by the applied buffering systems.

Due to the irreversibility of the transition we did not attempt to evaluate equilibrium thermodynamic parameters other than T_m. The latter value did not significantly vary with heating rate. Therefore it was not realistic to perform an analysis of the DSC curves on the basis of kinetic control of the transition. Inspection of the shape of the heat capacity function (Fig. 2, curve C) of the monomers seems to imply, however, a more complex transition behaviour than that expected from a simple two-state unfolding process. Such a conclusion is not unrealistic in view of the high molecular weight of the subunits of about 75,000 g mol^{-1}, possessing three domains with different folding motives, and the assumed maximal size of the cooperative folding unit of proteins, which is in the order of 200 amino acids [16,17].

In order to confirm the observed temperature stability of tarantula hemocyanin, light scattering experiments were performed (Fig. 3). To this end, the 24-mer hemocyanin was incubated at different temperatures between 20°C and 96°C. After cooling, the scattering intensites of the samples were measured at a wavelength of 400 nm at 20°C. In this experiment an irreversible decrease in the scattering intensity indicates a heat-induced denaturation of the hemocyanin. The intactness of the oxygen binding sites was also checked by recording the absorbance at 340 nm at 20°C. Fig. 3 shows that the quaternary structure of the hemocyanin as well as the oxygen binding sites are stable up to 80°C in the absence of Ca^{2+} and Mg^{2+}. The presence of these bivalent cations in physiological concentrations leads to an increase of the temperature stability of the 24-mer up to 90°C, thus confirming the results of the calorimetric experiment (Fig. 2).

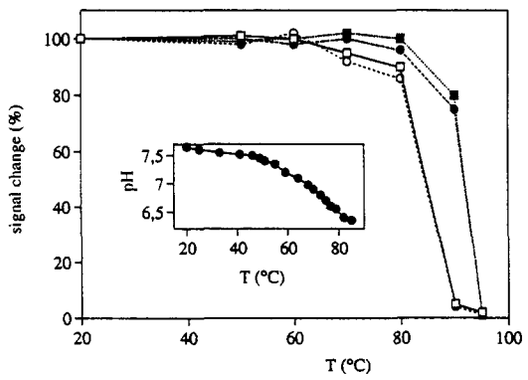

Fig. 3. Bivalent cations increase heat stability of tarantula hemocyanin. The hemocyanin was dissolved in 100 mM Tris-HCl buffer, pH 7.6 at 20°C, and exposed to different temperatures for 10 min. The experiments were performed either in presence (filled symbols) or in absence (open symbols) of 5 mM $CaCl_2$ and 5 mM $MgCl_2$. After cooling down to 20°C, light scattering intensities were measured at 400 nm (squares) as well as absorbances at 340 nm (circles). Mean values of three experiments are given. The standard deviation is about three times of the size of the symbol. The temperature-induced changes of the pH-value of the sample were measured simultaneously and are shown in the insert (filled circles).

During the heating of the sample, the concomittant change of the pH value was measured. Between 20°C and 85°C the pH value drops by 1.4 units, from 7.6 to 6.2 (Fig. 3), a result that is due to the strong temperature dependence of the pK value of Tris buffer. As shown by light scattering experiments (Fig. 3) this acidification of the solution does not result in a denaturation of the hemocyanin. This result is in accordance with analytical ultracentrifugation and oxygen binding experiments showing that tarantula hemocyanin is an intact 24-mer with cooperative oxygen-binding properties at least between pH-values of 5.0 and 9.0 [18]. This tolerance towards changes in the proton concentration of four orders of magnitude may also be a particular adaptation of tarantula hemocyanin. In vivo this hemocyanin has to transport oxygen under widely variing pH conditions, whereas for example human hemoglobin has to tolerate pH changes of only about 0.05 units [19,20]. The reason for the resistance to heat-induced pH-changes may lie in a temperature-induced change of the pK-value of histidine residues. This pK change compensates for the temperature-induced acidification of the blood of poikolothermic invertebrates and guarantees a constant degree of histidine protonation [21]. This prevents acid-induced denaturation of the corresponding proteins.

The intactness of a protein structure can also be tested by crossed immunogelelectrophoresis [13] since only intact epitopes on the surface of a protein can be recognized by conformation- specific antibodies. In addition, this method allows to discriminate between dissociated subunits and an intact quaternary structure. Fig. 4a–c shows the results obtained for the 24-meric hemocyanin. It is evident that an intact quaternary structure is retained after exposure to 80°C for 10 min. Only after an incubation at temperatures of 91°C and higher, some dissociation occurs. In contrast, subunit *e* is stable at temperatures up to 60°C (Fig. 4d,e) but it is denatured after incubation at 70°C (Fig. 4f). These results are in full accordance with the calorimetric data (Fig. 2).

In a further experiment, it was tested, if the oxygen binding properties of tarantula hemocyanin are changed with increasing time of heat incubation. To this end hemocyanin was incubated at 80°C for either 10 or 30 min, and after cooling oxygen

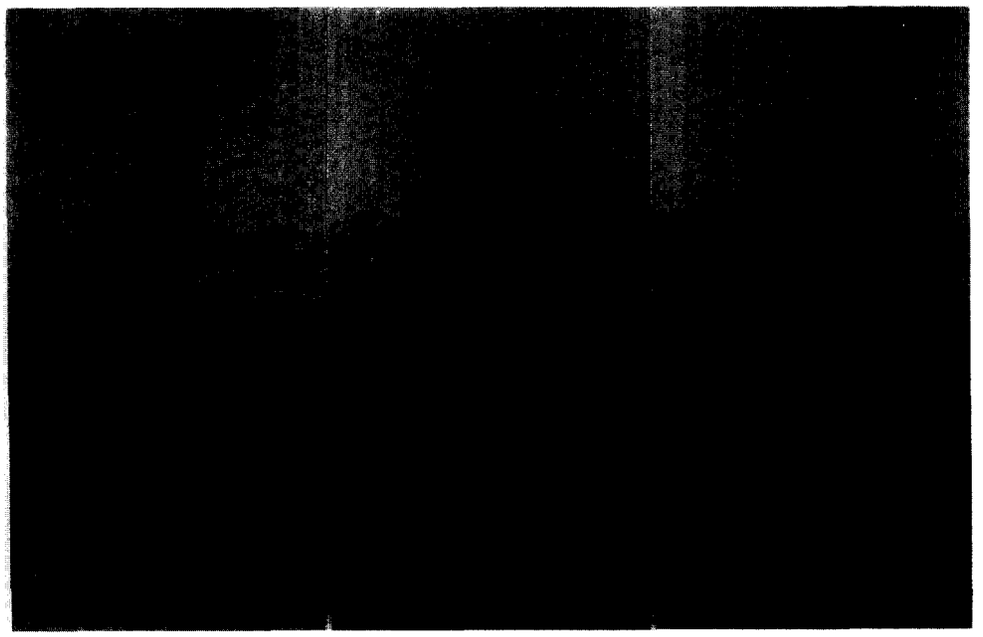

Fig. 4. Quaternary structure and epitopes of tarantula hemocyanin remain intact at 80°C. Crossed immuno-electrophoresis [2,13] was performed for the 24-meric hemocyanin (a–c) and for isolated subunit *e* (d–f). The experiments were performed at 20°C after a 10 min incubation of the hemocyanin at the particular temperature (20°C (a), 80°C (b), 91°C (c), 20°C (d), 60°C (e) and 70°C (f)). In case of the 24-meric hemocyanin the protein concentration was 2.0 g/l in 100 mM Tris-HCl buffer, pH 8.0 at 20°C, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. No $CaCl_2$ and $MgCl_2$ was present in the buffer in case of subunit *e*. The protein concentration was 0.6 g/l. In the second dimension the agarose bed was incubated with antibodies against dissociated 24-meric tarantula hemocyanin.

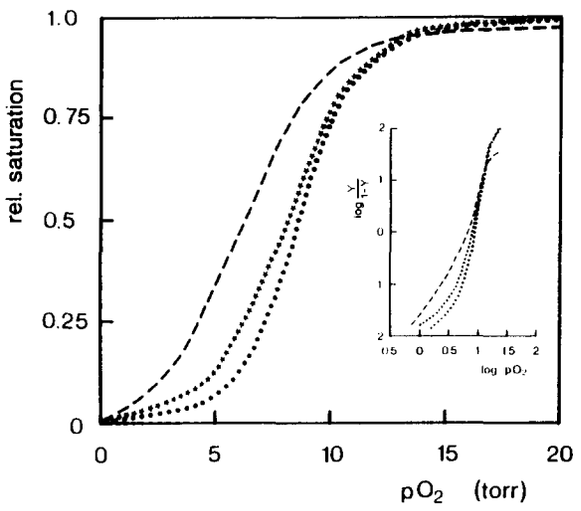

Fig. 5. Affinity and cooperativity of oxygen binding are only slightly modified after heat exposure. Oxygen binding curves with 24-meric hemocyanin were performed at 20°C after incubation at 80°C for 10 min (*), 30 min (−), and without previous heat exposure (●). (The insert shows the Hill plots of the oxygen binding curves). The hemocyanin concentration was 0.1 g/l in 100 mM Tris-HCl buffer, pH 8.0 at 20°C, containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$. After different times of heat exposure, the oxygen affinity was determined as follows: p_{50} = 8.5 Torr (at 0 min), p_{50} = 7.5 Torr (after 10 min), p_{50} = 6 Torr (after 30 min). The cooperativity of oxygen binding given as Hill coefficient at half oxygen saturation h_{50} was 6.2 (0 min), 6.0 (10 min) and 5.5 (30 min).

binding curves were recorded at 20°C (Fig. 5). After 10 min of heat exposure, oxygen binding was not changed significantly. Longer exposure to 80°C (30 min) leads to an increase of the oxygen affinity from 9 Torr to 6 Torr and a slight decrease of the Hill coefficient from 6.2 to 5.5, probably due to the dissociation of a small fraction of the oligomer.

Our results show that tarantula hemocyanin is an exceptional respiratory protein because it retains an intact structure and proper function after exposure to temperatures of up to 90°C and a concomitant decrease of the pH to values of about 6.2. The remarkable heat resistance is in contrast to the properties of the closely related but hexameric hemocyanin from *Palinurus vulgaris*, which denatures at almost 30°C lower temperatures between 60°C and 65°C [11]. The physiological significance of the different heat stabilities of tarantula and lobster hemocyanin can easily be rationalized. Whereas lobsters live in a marine habitat with moderate and only slightly changing temperature conditions, desert tarantulas have to cope with extreme temperature changes in their environment. Since tarantulas are poikilothermic animals, these changes are not buffered by an active regulation of the body temperature and can thus only be weakened, for example by behavioral adaptation. Therefore an extremely stable, and yet highly flexible, respiratory protein is crucial for the oxygen supply and thus the survival of tarantulas in their dreary habitat.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (De 414/4-1).

References

[1] Paul, R. and Effinger, R. (1990) in: Invertebrate Dioxygen Carriers (Preaux G. and Lontie R., Eds.) pp. 471–475, Leuven University Press.
[2] Lamy, J., Lamy, J., Weill, J., Markl, J., Schneider, H.-J. and Linzen B. (1979) Hoppe Seyler's Z. Physiol Chem. 360, 889–895.
[3] Markl, J., Kempter, B., Linzen, B., Bijlholt, M.M.C. and van Bruggen, E.F.J. (1981) Hoppe-Seyler's Z. Physiol. Chem. 362, 1631–1641.
[4] De Haas, F. and E.F.J. van Bruggen (1994) J. Mol. Biol. 237, 464–478.
[5] Decker H., J. Markl, R. Loewe, and B. Linzen (1979). Hoppe-Seyler's Z. Physiol. Chem. 360, 1505–1507.
[6] Loewe, R. (1978) J. Comp. Physiol. B 128, 161–168.
[7] Decker, H. and Sterner, R. (1990) J. Mol. Biol. 211, 281–293.
[8] van Holde, K.E. and Miller, K.I. (1982) Q. Rev. Biophys. 15, 1–129.
[9] Linzen, B., Soeter, N.M., Riggs, A., Schneider, H.-J., Schartau, W., Moore, M.D., Yokota, E., Behrens, P.Q., Nakashima, H., Takagi, T., Nemoto, T., Vereijken,J.M., Bak, H.J., Beintema, J.J., Volbeda, A., Gaykema, W.P.J. and Hol, W.G.J. (1985) Science 229, 519–524.
[10] Markl, J. and Decker, H. (1992) Adv. Env. Biochem. and Physiol. 13, 325–376.
[11] Guzman-Casado, M., Parody-Moreale, A., Mateo, P.L. and Sanchez-Ruiz, J.M. (1990) Eur. J. Biochem. 188, 181–185.
[12] Schneider, H.-J, Markl, J., Schartau, W. and Linzen, B. (1977) Hoppe-Seyler's Z. Physiol. Chem. 358, 1133–1141.
[13] Weeke, B. (1973) Crossed Immunelectrophoresis 1, 47–56.
[14] Markl, J., Strych, W., Schartau, W., Schneider, H.-J., Schöberl, P. and Linzen, B. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 639–650.
[15] Decker, H., Schmid, R., Markl, J. and Linzen, B. (1980) Hoppe-Seyler's, Z. Physiol. Chem. 361, 1707–1717.
[16] Dill, K.A. (1985) Biochemistry 24, 1501–1509.
[17] Hazes, B., Magnus, K.A., Bonaventura, C., Bonaventura, Dauter, Z., Kalk, K.H. and Hol, W.G. (1993) Prot. Sci. 2, 597–619.
[18] Decker, H. and Savel, A. (1990) in: Invertebrate Dioxygen Carriers (Preaux G. and Lontie R. Eds.), pp. 393–395, Leuven University Press.
[19] Angersbach, D. (1978) J. Comp. Physiol. 123, 113–125.
[20] Roughton, F.J.W. (1964) in: Handbook of Physiology (Fenn W.O. and Rahn H. Eds.) Vol. I, pp. 767–825, Am. Physiol. Soc., Washington DC.
[21] Reeves, R.B. (1972) Respiration Physiol. 14, 219–236.