A kinetic and equilibrium study of ligand binding to the monomeric and dimeric haem-containing globins of two chitons

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The radular muscles of the amphineuran molluscs *Amaurochiton glaucus* and *Sipharochiton pelleris* contain both a dimeric and a monomeric form of myoglobin. The dimeric form of the protein is composed of two polypeptide chains covalently linked to each other via one or more disulphide bonds. The dimeric protein shows co-operative O₂-binding curves. Kinetic investigations indicate that CO binding is co-operative in the dimeric protein, subsequent to full photolysis, but mono-exponential following 10 % photolysis. O₂ recombination following part photolysis is mono-exponential in the dimeric form, whereas O₂ dissociation kinetics indicates the presence of chain heterogeneity. The monomeric form of the protein exhibits mono-exponential time courses in all the experimental situations explored. Although the rate constants associated with the reactions of individual dimer and monomer molecular species are very different, the two species of chiton investigated show remarkably similar properties when compared with each other. All the reactions studied are pH-independent in the range pH 6–8. Amino acid analysis indicates that the monomeric units that combine to form the dimeric species are not identical with the naturally occurring monomeric form. A comparison is made between the chiton myoglobins and other similar O₂-binding proteins.

INTRODUCTION

In mammalian systems O₂ transport invariably involves the uptake of O₂ by tetrameric haemoglobin and later release of O₂ to monomeric myoglobins. The co-operative functioning of the haemoglobin system has long been identified with the existence, within the haemoglobin tetramer, of a pair of heterodimers [1]. A number of investigators have forwarded theoretical justification for the necessity of heterodimers as a prerequisite of co-operative function [2–4]. The heterodimer itself, however, has been shown to be devoid of co-operative function [5,6]. In lower animals clear evidence exists that heterogeneity of subunits is not a pre-requisite for co-operative O₂ binding. A particular case in point is that of the haemoglobin system of the arcid clams, which contains a co-operative dimeric haemoglobin composed of identical monomeric subunits [7–11].

In other invertebrates dimeric myoglobins have been found that show co-operative function [12].

In the case of the chitons both monomeric and dimeric myoglobins have been reported as being present simultaneously in the radular muscle [13,14]. It is with the view to obtaining a clearer insight into the nature of co-operative functioning within homodimers that we have undertaken a study of the structure, equilibrium and kinetic properties of the myoglobins from two species of chiton. Whereas for monomeric myoglobins a comparison between both kinetic and equilibrium data is possible in many species, only limited comparisons can be made within the dimeric systems for as yet only limited kinetic data are available on co-operative homodimeric myoglobin systems [15,16].

MATERIALS AND METHODS

About 3000 chitons were collected intertidally from the Waitamata Harbour of Auckland and identified as

either *Amaurochiton glaucus* or *Sipharochiton pelleris*. The radular muscles were dissected and dropped directly into liquid N₂. The frozen tissue was then processed essentially according to the method of Terwiliger [14] with the exception that cold (4 °C) 0.05 M-Tris/HCl buffer, pH 8.0, supplemented with 1 mM-EDTA was used in the procedure in order to minimize oxidation of the myoglobin [17]. Exclusion chromatography and electrophoresis were performed as previously described [18–20].

Globin chain separation by reversed phase h.p.l.c. was based on the principles of Congote [21] with the use of apparatus described previously [22]. O₂-binding curves on the purified fractions were obtained by using a modified thin-layer instrument in which continuous curves were run from the deoxy to the oxy form in approx. 1 h. Residual dynamic errors were corrected by measuring a number of static points for each curve [23].

Amino acid analysis and hydrolysis of proteins were performed by using methods previously described [24].

Stopped-flow and flash-photolysis experiments were performed with apparatus previously detailed [25,26]. Dithionite-treated and gas-equilibrated solutions were prepared in the manner described previously [27].

The two-state analysis of the O₂-binding curves obtained for the dimeric species employed the equations appropriate to a dimeric system given by Levitzki [28].

RESULTS

After disruption of the radular muscle, sequential (NH₄)₂SO₄ precipitation yielded a myoglobin-rich fraction at 70 % saturation. Chromatography of the myoglobin-rich fraction on a calibrated Sephadex G-75 column yielded three peaks. A non-haem-containing protein band appeared close to the void volume of the column followed by two myoglobin peaks at retention volumes indicative of *M_r* values of 35000 and 17000. The

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Fig. 1. SDS/polyacrylamide-gel-electrophoretic separation of myoglobin species

Protein samples were run in SDS-containing 15% polyacrylamide gels and the resulting bands were stained with Coomassie Brilliant Blue. The lower trace illustrates the separation of monomeric and dimeric species (M and D respectively), and the upper trace illustrates the electrophoretic patterns obtained for dithiothreitol-treated dimer (—) and dithiothreitol + *N*-ethylmaleimide-treated dimer (-----). Absorbances were measured at 632.8 nm by using a laser gel scanner. * shows marker band.

Table 1. Amino acid compositions of chiton globins

Amino acid	Amino acid composition (mol of residue/100 mol)			
	<i>Sipharochiton</i> globins		<i>Amaurochiton</i> globins	
	Monomer	Separated chain of dimer	Monomer	Separated chain of dimer
Asx	10.0	9.3	9.7	8.7
Glx	8.8	9.5	9.3	10.4
Ser	4.4	8.0	4.7	7.7
Gly	14.6	11.9	12.6	12.0
His	1.0	1.7	0.7	1.5
Arg	1.5	4.5	1.4	4.0
Thr	3.3	3.2	4.7	3.0
Ala	16.3	6.5	14.7	7.5
Pro	4.1	3.4	4.4	3.0
Tyr	1.5	1.0	1.8	1.4
Val	5.0	7.5	5.3	6.1
Met	1.0	1.8	1.5	4.2
Ile	7.6	6.5	8.0	7.4
Leu	6.6	7.5	5.7	6.9
Phe	3.6	6.7	3.5	6.8
Lys	10.6	11.4	11.5	10.2

relative proportions of these two fractions were essentially constant over a number of preparations. Electrophoresis of the two fractions on SDS/polyacrylamide gels showed that both fractions retained the same M_r values as those exhibited on Sephadex G-75 chromatography. Treatment of the 35000- M_r fraction with dithiothreitol before electrophoresis led to a somewhat diffuse band with an apparent M_r of 17000, whereas prior treatment with dithiothreitol and *N*-ethylmaleimide

Fig. 2. Hill plots of O_2 binding by myoglobin

The Hill plots of O_2 binding to the monomeric (a) and dimeric (b) myoglobins of *Amaurochiton* (○) and *Sipharochiton* (●) are shown. In (a) the line is shown for $n = 1$. In (b) the asymptotes of unit slope are indicated for the fitted values of K_R and K_T . The line (b) represents the best-fit curve to the data of a two-state model with $L = 15$.

produced a single sharp band with an apparent M_r of 17000 (Fig. 1).

Both monomeric and dimeric species obtained by Sephadex G-75 chromatography, when chromatographed by reversed-phase h.p.l.c., showed one major band (approx. 90%) and a more hydrophobic minor band (approx. 10%). Results of the amino acid analysis of the monomeric myoglobins and the separated chains of the dimeric myoglobins are shown in Table 1.

The O_2 -binding curves obtained for the monomeric and dimeric fractions showed only minor intraspecies differences. The monomeric forms exhibited non-co-operative hyperbolic binding curves with Hill coefficients of $1.03 (\pm 0.1)$ and $0.85 (\pm 0.09)$ and p_{50} values of $0.95 (\pm 0.18)$ mmHg and $0.63 (\pm 0.07)$ mmHg for *Sipharochiton* and *Amaurochiton* respectively. The dimeric proteins exhibited co-operative sigmoidal binding curves with Hill coefficients of $1.35 (\pm 0.07)$ and $1.37 (\pm 0.06)$ and p_{50} values of $3.5 (\pm 0.47)$ mmHg and 2.76

Fig. 3. Effect of CO concentration on the rate of its recombination with myoglobin

The rate of recombination of the various myoglobins with CO subsequent to flash photolysis is shown for *Amaurochiton* monomer (■) and dimer (□) and *Sipharochiton* monomer (●) and dimer (○). The reactions were monitored at 437 nm in 0.05 M-Tris/HCl buffer, pH 8, containing 1 mM-EDTA at 20 °C. In the case of the dimers the rate is that obtained from an exponential fit of the last 90 % of the time course.

(± 0.48) mmHg for *Sipharochiton* and *Amaurochiton* respectively (Figs. 2a and 2b).

The recombination of the monomeric proteins with CO subsequent to flash photolysis occurred in a single-exponential process with an associated second-order rate constant of $5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$. Full photolysis of the dimeric material on the other hand produced accelerating time courses in which most (approx. 90 %) of the latter portion of the time course followed an exponential pattern with an associated second-order rate constant of $2 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 3). Partial photolysis of the dimeric CO complex (approx. 10 %) yielded single-exponential time courses with a second-order rate constant of $1.8 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Figs. 4a, 4b and 4c). O_2 -dissociation rates measured by rapid anaerobic mixing with an excess of dithionite produced single-exponential time courses for the monomeric myoglobins with a first-order rate constant of 120 s^{-1} . Deoxygenation of the dimeric species, however, followed a complex time course consisting of two exponential processes with associated rate constants of 100 s^{-1} and 2 s^{-1} . At a wavelength of 435 nm the two processes contributed equally to the overall absorbance change observed. Conventional 200 J flash lamp photo-

Fig. 4. Residual data of fitted exponential time courses for the recombination of CO with myoglobin

The residual data are shown resulting from the subtraction of the experimental data points from that obtained from the best-fit single exponential. In the case of the data obtained for full photolysis of the monomer (a) and 10 % photolysis of the dimer (c) all the experimental data points were employed in the fit. In the case of full photolysis of the dimer (b) the best-fit exponential was to the last 90 % of the time course. Experimental conditions were as indicated in Fig. 3 legend.

lysis produced 8 % dissociation of the monomeric oxy complex and 13 % dissociation of the dimeric oxy complex. In both cases this partial photolysis led to single-exponential recombination time courses, with the monomeric protein exhibiting a second-order rate constant of $1.5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$ and the dimeric protein a rate constant of $2 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 5).

In all the kinetic studies undertaken the nature of the time courses and the observed rates of reaction were found to be pH-independent in the region pH 6–8.

DISCUSSION

From the combined results of molecular-mass exclusion chromatography and electrophoresis studies it is clear that the *Amaurochiton* and *Sipharochiton* both possess monomeric and dimeric myoglobins in their radular muscles. The dimer appears to contain identical monomeric units that are linked via one or more disulphide linkages. Dithiothreitol alone appears able to monomerize the dimer, although the rather broad band

Fig. 5. Effect of O_2 concentration on the rate of recombination with myoglobin

The rates of recombination of *Amaurochiton* monomer (○) and dimer (●) and *Sipharochiton* monomer (□) and dimer (■) subsequent to partial photolysis (10%) of the oxy complex are shown. The reactions were monitored at 435 nm in 0.05 M-Tris/HCl buffer, pH 8.0, containing 1 mM-EDTA at 20 °C.

observed on electrophoresis subsequent to this treatment may well indicate partial reoxidation during the course of the experiment. Alkylation of the reduced disulphide bonds clearly leads to a monomeric species. These findings are in accord with those previously made on the larger North American chitons [14]. The amino acid compositions of the various peptides constituting the myoglobin system of the radular muscle of the chitons are also worthy of note. The dimeric species are relatively rich in serine, arginine and phenylalanine, and the monomers are relatively rich in alanine and glycine. This

general pattern in composition is very similar to that previously reported in some other chitons [14]. It is also significant that amino acid analysis of the minor peaks associated with each of the major myoglobin peaks gave results indistinguishable from those of the associated major peaks. As our analysis does not distinguish between the amide and parent acid forms of aspartic acid and glutamic acid, we therefore assume that the minor peaks represent the presence of minor de-amidated forms of the parent myoglobins.

The functional characteristics of the monomeric chiton myoglobins are typical of those seen for other monomeric myoglobins, showing high affinity, non-co-operative binding and insensitivity to pH (see Table 2). The binding parameters associated with the dimeric species fall within the range observed for other homodimeric systems, which exhibit pH-independent Hill coefficients ranging from 1.2 to 1.5 and O_2 affinities ranging from 0.7 mmHg to 17 mmHg [7,9,10,15,29]. Further analysis of the binding curves for dimeric myoglobin within the framework of the two-state model of co-operativity [30] yields values of 1 mmHg for K_R and 13 mmHg for K_T with an associated allosteric equilibrium constant (L) of 15, indicating that the equilibrium between the relaxed and tense unliganded forms only slightly favours the less-reactive T state. These parameters indicate that the concerted switch from predominantly T state to predominantly R state occurs almost exactly at the stage of mono-ligation.

In the total-photolysis experiments, used to monitor CO recombination, the presence of accelerating time courses is in accord with the presence of a co-operative system in which the initially formed unliganded R state species undergoes a transition to the unliganded T state at a rate faster than that of ligand recombination [31,32]. In the case of partial photolysis (approx. 10%) the position of the concerted switch between T state and R state predicts that photolysis would yield predominantly partially liganded R states, which would then exhibit simple recombination time courses characteristic of ligand binding to the isolated R state, as is observed experimentally [32].

In the case of O_2 recombination kinetics sufficient

Table 2. Kinetic constants for some myoglobins

l_{on} is the rate of CO binding, l_{off} is the rate of CO dissociation, k_{on} is the rate of O_2 binding and k_{off} is the rate of O_2 dissociation.

Protein	$10^{-6} \times l_{on}$ ($M^{-1} \cdot s^{-1}$)	l_{off} (s^{-1})	$10^{-7} \times k_{on}$ ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	References
Liver fluke	110	0.65	30	30	[34]
Sperm whale	0.65	0.015	1.9	10	[38]
Glycera	22	0.055	19	2800	[35]
Ascaris	17	0.018	0.1	0.004	[36]
Aplysia	0.5	0.02	1.5	70	[38]
Chironomus	27	0.095	30	218	[37]
<i>Amaurochiton</i> (monomer)	6.5	—	12.5	120	
<i>Sipharochiton</i> (monomer)	4.3	—	12.5	117	
Sea-cucumber (dimer)	0.002 (0.01)	—	1.3 (0.045)	18 (3.6)*	[15,16]
<i>Amaurochiton</i> (dimer)	1.6	—	2.13	91 (2.9)*	
<i>Sipharochiton</i> (dimer)	2.1	—	2.17	104 (1.6)*	

* Values for two types of chains.

photolytic energy was available to allow only partial photolysis (approx. 10%), and therefore in these experiments only binding to the R state could be expected. The description of the kinetic findings given above assumes that the haem moieties within the dimer are kinetically equivalent. This appears to be the case in association of the myoglobins with ligand. However, chain heterogeneity does exist in the process of O₂ dissociation from the dimeric myoglobins, as illustrated by the presence of double-exponential time courses at 435 nm. This situation has been identified previously in haemoglobins from a number of species [33], but represents a novel finding in a homodimer. A comparison of the kinetic data obtained for chiton myoglobins with those of other myoglobin species is shown in Table 2.

Whereas vertebrate myoglobin is monomeric and haemoglobin tetrameric, molluscan myoglobins exist that are monomeric, dimeric and tetrameric [12]. It has been proposed that the level of molecular complexity seen in a particular mollusc is associated with the evolutionary state of the species concerned [33]. However, this interpretation has been questioned [12]. In the case of chitons both monomeric and dimeric proteins are present in the same muscle, and it is tempting to propose that this relates not to their evolutionary state but to the fact that these creatures are intertidal and so require O₂-binding systems that are optimized for both aquatic and aerobic environments, a requirement that can be met by the simultaneous presence of two O₂-binding systems. At present only four molluscan myoglobins have been sequenced, of which only two are dimeric forms [39–42]. On the basis of recent studies it seems likely that the subunits of the dimeric chiton myoglobins will show the usual globin fold [43]. The question as to whether the subunits in the dimer bind in the unusual 'back-to-back' manner seen in the clam myoglobin [8] must await X-ray crystallographic analysis. The major remaining question concerning the covalently linked dimeric systems is what conformational changes are associated with the co-operative functioning of these proteins. That significant structural changes can be associated with covalently linked subunits is evidenced from studies on 2-nor-formylpyridoxal 5'-phosphate-linked human haemoglobin, in which the β -chains are covalently bonded. In this situation the tetrameric system retains a high degree of co-operativity and shows a lowered O₂ affinity [44], although no detailed investigations have been made on the conformational changes associated with its allosteric operation.

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REFERENCES

- Perutz, M. F. (1979) *Annu. Rev. Biochem.* **48**, 327–386
- Ferreira, R. & Jacchieri, S. G. (1984) *J. Theor. Biol.* **108**, 191–201
- Weber, G. (1982) *Nature (London)* **300**, 603–607
- Peller, L. (1982) *Nature (London)* **300**, 661–662
- Hewitt, J. A., Kilmartin, J. V., Ten Eyck, L. F. & Perutz, M. F. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 203–207
- Ackers, G. K., Johnson, M. L., Mills, F. C. & Ip, S. H. C. (1976) *Biochem. Biophys. Res. Commun.* **69**, 135–142
- Chiancone, E., Vecchini, P., Vercili, D., Ascoli, F. & Antonini, E. (1981) *J. Mol. Biol.* **152**, 577–592
- Royer, W. E., Love, W. & Fenderson, F. F. (1985) *Nature (London)* **316**, 277–280
- Freadman, M. A. & Mangum, C. P. (1976) *Comp. Biochem. Physiol.* **53**, 173–179
- San George, R. C. & Nagel, R. L. (1985) *J. Biol. Chem.* **260**, 4331–4337
- Furuta, H. & Kajita, A. (1983) *Biochemistry* **22**, 917–922
- Terwilliger, R. C. & Terwilliger, N. B. (1985) *Comp. Biochem. Physiol.* **81**, 255–261
- Terwilliger, R. C. & Read, K. R. H. (1969) *Comp. Biochem. Physiol.* **29**, 551–560
- Terwilliger, R. C. & Read, K. R. H. (1970) *Int. J. Biochem.* **1**, 281–291
- Steinmeier, R. C. & Parkhurst, L. J. (1979) *Biochemistry* **18**, 4645–4651
- Parkhurst, L. J. & Steinmeier, R. C. (1979) *Biochemistry* **18**, 4651–4655
- Shikama, K. & Sugawara, Y. (1978) *Eur. J. Biochem.* **91**, 407–413
- Andrews, P. (1964) *Biochem. J.* **91**, 222–233
- Reisfield, R. A., Lewis, U. J. & Williams, D. E. (1962) *Nature (London)* **195**, 281–283
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
- Congote, L. F. (1981) in *Advances in Haemoglobin Analysis* (Hanash, S. M. & Brewer, G. J., eds.), pp. 39–45, Alan R. Liss, New York
- Tetens, V., Brittain, T., Christie, D. L., Robb, J. & Wells, R. M. G. (1984) *Comp. Biochem. Physiol.* **79**, 119–123
- Lapennas, G. N., Colacino, J. M. & Bonaventura, J. (1981) *Methods Enzymol.* **76**, 449–470
- Mathews, A. J. & Brittain, T. (1986) *Biochem. J.* **240**, 181–187
- Brittain, T. (1980) *Biochem. J.* **187**, 803–804
- Brittain, T. (1984) *Biochem. J.* **221**, 561–568
- Blackmore, R. & Brittain, T. (1986) *Biochem. J.* **233**, 553–557
- Levitzki, A. (1978) *Quantitative Aspects of Allosteric Mechanisms*, pp. 1–100, Springer-Verlag, Berlin
- Terwilliger, R. C. & Read, K. R. H. (1971) *Int. J. Biochem.* **2**, 253–261
- Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol.* **12**, 88–118
- Dickinson, F. M. & Gibson, Q. H. (1981) *Biochem. J.* **197**, 437–446
- Hopfield, J. J., Shulman, R. G. & Ogawa, S. (1971) *J. Mol. Biol.* **61**, 425–433
- Olson, U. S., Anderson, M. E. & Gibson, Q. H. (1971) *J. Biol. Chem.* **246**, 5919–5923
- Di Iorio, E. E., Meier, U. T., Smit, J. D. G. & Winterhalter, K. H. (1985) *J. Biol. Chem.* **260**, 2160–2164
- Parkhurst, L. J., Sima, P. & Goss, D. J. (1980) *Biochemistry* **19**, 2688–2692
- Traylor, T. G. & Traylor, P. S. (1982) *Annu. Rev. Biophys. Bioeng.* **11**, 105–127
- Amiconi, G., Antonini, E., Brunori, M., Formanek, H. & Huber, R. (1972) *Eur. J. Biochem.* **31**, 52–58
- Antonini, E. & Brunori, M. (1971) *Haemoglobin and Myoglobin in their Reactions with Ligands*, pp. 219–227, North-Holland, Amsterdam

39. Tentori, L., Vivaldi, G., Carta, S., Marinuci, M., Massa, A., Antonini, E. & Brunori, M. (1973) *Int. J. Pept. Protein Res.* **5**, 182–200
40. Suzuki, T., Takagai, T. & Shikawa, K. (1981) *Biochim. Biophys. Acta* **669**, 79–83
41. Takagai, T., Tobita, M. & Shikawa, K. (1983) *Biochim. Biophys. Acta* **745**, 32–36
42. Bonner, A. S. & Laursen, R. A. (1977) *FEBS Lett.* **73**, 201–203
43. Bashford, D., Chothia, C. & Lesk, A. M. (1987) *J. Mol. Biol.* **196**, 199–216
44. Benesch, R. & Benesch, R. E. (1981) *Methods Enzymol.* **76**, 147–159

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