Aust. J. Biol. Sci., 1981, **34**, 5–10

Myoglobins of Cartilaginous Fishes III.* Amino Acid Sequence of Myoglobin of the Shark *Galeorhinus australis*

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Abstract

Myoglobin isolated from the red muscle of the school shark *Galeorhinus australis* was purified by gel filtration and ion-exchange chromatography. The amino acid sequence was determined following digestion with trypsin and purification of the peptides by paper ionophoresis and chromatography. Sequences of purified peptides were determined by the dansyl–Edman procedure and the peptides aligned by homology with the sequence of the myoglobin of the gummy shark *Mustelus antarcticus*. The two myoglobin sequences showed a marked similarity (16 differences), but both sequences showed approximately the same number of differences (68) from myoglobin of the Port Jackson shark *Heterodontus portusjacksoni*. There are 19 residues unique to the three shark myoglobin sequences.

As found with other fish myoglobins there are 148 residues with deletions of four residues at the amino terminal end as well as one residue in the CD region. The amino terminal residue is acetylated. The distal E7 histidine residue was found to be replaced by glutamine, as only previously reported for the myoglobin sequence of gummy shark.

Introduction

Previous papers of this series (Fisher and Thompson 1979; Fisher *et al.* 1980) presented the amino acid sequences of myoglobin from the 'primitive' shark *Heterodontus portusjacksoni* and the gummy shark *Mustelus antarcticus* (Triakidae), a representative of 'modern' sharks (Romer 1966). Sixty-eight differences between the myoglobin sequences of these two sharks were seen. The sequence of amino acids in another 'modern' shark myoglobin, from the school shark *Galeorhinus australis* (Carcharhinidae), is presented in this paper. It is one of the most abundant of Australian sharks, commonly reaching a length of 1·2–1·5 m. The body is long and somewhat slender with a pointed, slightly flattened snout with rows of teeth.

The amino acid sequence of the myoglobin from the school shark was deduced from sequences of the purified tryptic peptides determined by the dansyl–Edman procedure. The peptides could be placed in order from the strong homology to the sequence of myoglobin from the gummy shark (Fisher *et al.* 1980).

The sequence has been compared with the sequences of myoglobins from other sharks and that of a bony fish.

* Part II, *Aust. J. Biol. Sci.*, 1980, **33**, 153–67.

Materials and Methods

The methods of isolation of the school shark myoglobin, fractionation of apomyoglobin, fractionation of enzyme digests, sequence procedures and determination of the blocking group on the amino terminal of the myoglobin chain were substantially the same as previously described for gummy shark myoglobin (Fisher *et al.* 1980).

The major tryptic digest investigated, where chymotryptic-like splits were found, was the result of digestion for 16 h at 37°C in 1% (w/v) ammonium bicarbonate. For the isolation of the insoluble tryptic peptides a 3 h digestion was used.

Results

Isolation of Shark Myoglobin

Gel filtration of the crude extract from the red muscle of the school shark on Sephadex G75 (Fisher *et al.* 1980) gave a clearly separated protein fraction containing myoglobin. Fractionation of the apomyoglobin on CM-cellulose in urea–thiol buffers, as previously described, removed any contaminating proteins.

Amino Acid Composition

The amino acid composition of school shark myoglobin determined on 6M HCl hydrolysates for 24–96 h, with corrections for destruction of serine and threonine and slow liberation of hydrophobic residues, showed only 13 differences from the amino acid composition of gummy shark myoglobin, mostly involving single changes in particular amino acids. The amino acid composition agreed with the sequence except for a deficiency of 0·9 residues of alanine.

Determination of the Blocking Group on the Amino Terminus of Myoglobin

A chymotryptic *N*-terminal peptide that was not adsorbed on sulfonated polystyrene was purified by paper electrophoresis, pH 6·4. The peptide stained positively for tryptophan with Ehrlich's reagent (Smith 1953) and gave only aspartic acid and alanine on acid hydrolysis. A mass spectrograph trace of this methylated peptide (Fisher *et al.* 1980) gave a prominent peak at mass 114 confirming acetyl-alanine, and a total mass +1 of 461 showing the sequence to be acetyl-Ala-Asp-Trp. No unblocked *N*-terminal peptide was found, and in this respect school shark myoglobin differed from gummy shark myoglobin in which both the acetylated peptide and a small amount of unblocked peptide were detected by Ehrlich's reagent.

Amino Acid Sequence of Peptides

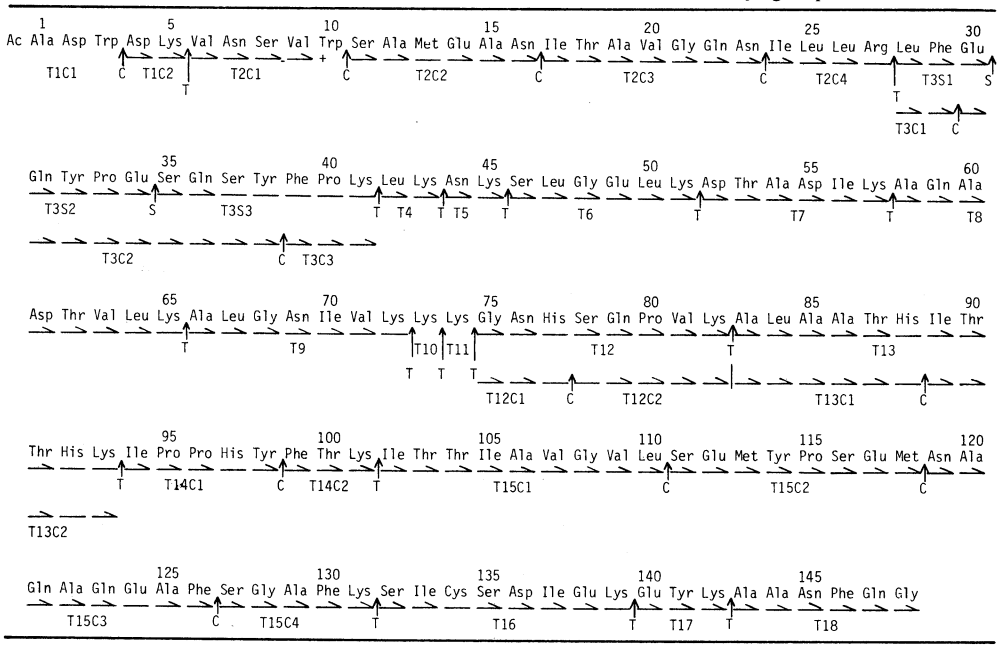
Amino acid compositions of tryptic peptides, including *N*- and *C*-terminal portions of some peptides split by chymotryptic-like activity, were determined. Satisfactory analyses were obtained for most tryptic peptides. Of the two large insoluble peptides T2 and T15, containing 22 and 30 residues respectively, T2 was never isolated intact and T15 was partially purified after solution of the insoluble 'core' in pH 1·9 buffer (6·2% formic acid) and paper electrophoresis at pH 1·9. The sequences of the tryptic peptides are shown in Table 1. Also shown in Table 1 is the designation and sequence of peptides obtained by secondary splits by chymotrypsin or chymotryptic-like activity, or by staphylococcal protease digestion.

Amino Acid Sequence of School Shark Myoglobin

The complete amino acid sequence of school shark myoglobin is shown in Table 1 with the amide groups assigned to particular residues. These assignments were made predominantly by ionophoretic mobility at pH 6·4.* The tryptic peptides were aligned by homology with the sequence of gummy shark myoglobin. This could be done without ambiguity because of the strong similarity in amino acid sequence.

Table 1. Amino acid sequence of myoglobin of the school shark

Residues are numbered sequentially from the *N*-terminal residue. Vertical arrows indicate bonds digested by trypsin (T), chymotrypsin (C) or staphylococcal protease (S). Residues identified by the dansyl–Edman procedure are underlined by an arrow. If no horizontal arrow is shown, the residue did not give a clear result. Ac represents an acetyl group



In order to confirm residues of the sequence which were different from any of the previous fish myoglobin sequences, for example residues 13, 16, 37–38, 42, 76, 108, 132, particular attention was paid to the analysis and sequence of the relevant tryptic peptide, or, alternatively, peptides from a chymotryptic or cyanogen bromide digest were isolated. Thus from a cyanogen bromide digest of the insoluble tryptic peptides a fragment was isolated corresponding to residues 6–13 with the analysis $Asp_{0.9}Ser_{1.7}Hse_{0.7}Ala_{1.0}Val_{1.3}Trp$ (Ehrlich positive). This assigned methionine to position 13, which in all previously sequenced myoglobins has been a valyl residue. Peptides T2C2, T3C2 and T12C1 resulting from chymotryptic-like specificity during tryptic digestion had ionophoretic mobilities and sequences that confirmed the allocations for residues 16, 37–38 and 76 respectively.

* Supplementary data to this paper are deposited with and can be obtained from the Editor-in-Chief, Editorial and Publications Service, CSIRO, P.O. Box 89, East Melbourne, Vic. 3002. The data deposited contain the following material: amino acid analysis of soluble tryptic peptides or fragments of them; peptides and their net charge used for assignment of side-chain amide and acidic groups.

Table 2. Amino acid sequences of human and fish myoglobins

Helical regions A–H and interhelical regions NA, AB, CD ... etc. are numbered. One-letter code and alignment follow Dayhoff (1972). Sequence of human myoglobin is given in full; dashes indicate deletions or insertions in longer globins. Dots indicate identity with the residue in human myoglobin. Ac represents an acetyl group. References are given in the text

	NA A ... A B ... B C ... C CD ... CD D
Myoglobin	1 2 3 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 1 2 3 4 5 6 7 1 2 3 4 5 6 7 8 1 2 3
Human	G - L S D G E W Q L V L N V W G K V E A D I P G H G Q E V L I R L F K G H P E T L E K F D K F K H L K S E D
Tuna	AcA D F D A . . K C . . P . . . . . Y T T M . G L . . T . . . . . E . . . . Q K L . P . . A G - I A Q A
Shark	
Heterodontus sp.	AcT . . E H . N K . . A V . . P . . . A V . L A I . L . . . . . E . K . . K D L . P . . . E - I P V Q
Mustelus sp.	AcV D . E K . N S . . S A . . S . L T A I . . N I . L . . . . E Q Y . . S Q N H . P . . . N - K . L G
Galeorhinus sp.	AcA D . D K . N S . . S A M . . N . T A V . . N I . L . . . . E Q Y . . S Q S Y . P . L . N - K . L G
	D DE ... E EF ... EF F ... F FG ... FG G
Myoglobin	4 5 6 7 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 1 2 3 4 - 5 6 7 8 1 2 3 4 5 6 7 8 9 1 2 3 4 5 1 2 3 4 5 6 7 8
Human	E M K A S E D L K K H G A T V L - T A L G G I L K K K G H - H E A E I K P L A Q S H A T K H K I P V K Y L E F I
Tuna	D I A G N A A I S A . . . . K K . . E L . . A . . S . . A . I L . . . . N . . . . I N N F K L .
Shark	
Heterodontus sp.	Q L G N N . . . R . . . V . . . . . R . . . . N . . . . Q . . K . . S T N V . E . . D T . I N . . . . . P . N F V L .
Mustelus sp.	. L . D T A . I . A Q A D T . . . S . . . . N . V . . . . S . . S Q P V . A . . A T . I . T . . . . P H . F T K .
Galeorhinus sp.	. L . D T A . I . A Q A D T . . . K . . . . N . V . . . . N . . S Q P V . A . . A T . I . T . . . . P H . F T K .
	G ... G GH ... GH H ... H HC
Myoglobin	9 10 11 12 13 14 15 16 17 18 19 1 2 3 4 5 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 1 2 3 4
Human	S E C I I Q V L Q S K H P G D F G A D A Q G A M N K A L E L F R K D M A S N Y K E L G F Q G
Tuna	. . V L V K . M H E . A G L . - - - . G G . T . L R N V M G I I I A . L E A . . . . S .
Shark	
Heterodontus sp.	T N I A V K . . T E M Y . S . M T G P M . E S F S . V F T V I C S . L E T L . . . A N . . .
Mustelus sp.	T T I A V D . . S E M Y . S E M N . Q V . A . F S G . F K I I C S . I E K E . . A A N . . .
Galeorhinus sp.	T T I A V G . . S E M Y . S E M N . Q . . E . F S G . F K S I C S . I E K E . . A A N . . .

A chymotryptic digest of myoglobin was used to isolate some additional peptides with *N*-terminal phenylalanine representing the peptides 39–42 and 39–44. Also from the chymotryptic digest a peptide with a glycyl *N*-terminal residue corresponding to residues 70–76 confirmed the presence of three lysyl residues. Additional peptides obtained by chymotryptic digestion, or by chymotryptic or cyanogen bromide digestion of maleylated globin fractions and not described here, gave other peptides that were analysed and partially sequenced to confirm the sequence shown in Table 1.

Discussion

When the sequence of myoglobin from the school shark *Galeorhinus australis* is compared with the sequences of the myoglobins of two other sharks and tuna (cf. Fisher *et al.* 1980) there is similarity in the acetylation of the *N*-terminal residue, in the 148 residues in the chain resulting from the shortened length of chain at the amino terminal end, in four residues missing compared with mammalian myoglobins, and in the deletion of a residue corresponding to CD7 (leucine residue 49 in other myoglobins) in the folded myoglobin structure.

The differences in amino acid sequence are shown in Table 2. There are only 16 differences between the sequences of myoglobin from the gummy shark *Mustelus antarcticus* and the school shark *Galeorhinus australis*, indicating a relatively recent radiation of the two families represented. The differences in amino acid sequences for both these sharks from the myoglobin sequence of the Port Jackson shark *Heterodontus portusjacksoni* are large, 68 and 69 respectively, indicating a relatively long period since divergence from it. When the one myoglobin sequence from a bony fish, the yellowfin tuna (Brown *et al.* 1979; W. D. Brown, personal communication), is compared with the sequences of the myoglobins from the Port Jackson, gummy and school sharks there are even more differences in sequence 83, 90 and 91 respectively.

When all the fish myoglobin sequences are compared (Table 2), there are only four residues, proline CD2, phenylalanine G5, isoleucine H15, and glutamic acid H20, that are identical in the fish and different from the residue common in 29 myoglobins of other animals (Fisher *et al.* 1980). There are, however, 19 residues unique to the three shark myoglobin sequences.

The distal E7 histidine residue was found to be replaced by glutamine, as only previously reported for the myoglobin sequence of gummy shark. As in the case of gummy shark myoglobin (Fisher *et al.* 1980) the deletions at the amino terminus resulting in the loss of residue A2, and the presence of amino acids with uncharged side-chains at positions E2, G6 and G19 and their replacement during evolutionary divergence by residues with charged side-chains in more recent classes of animals, has resulted in a loss of four of the seven salt-bridges that help to stabilize the secondary and tertiary structure in the myoglobins of animals of more recent classes.

Acknowledgments

This work was supported in part by the Australian Research Grants Committee. We are indebted to Drs Terry Gorman and Ken Graham, N.S.W. State Fisheries, for supplying the school sharks; to Mr R. G. Mann for the amino acid analyses, and to Dr A. M. Duffield for the mass spectrograph analyses.

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Manuscript received 1 September 1980, accepted 5 November 1980