INTRAMOLECULAR LOCATION OF THE SUBUNITS OF *ANDROCTONUS AUSTRALIS* HEMOCYANIN

Jean LAMY, Josette LAMY, Michèle LECLERC, Pierre-Yves SIZARET and Jacques WEILL
Laboratoire de Biochimie, Faculté de Pharmacie, 37032 Tours Cedex, France

Received 24 January 1980

1. Introduction

Arthropod hemocyanins (Hc) are large molecular mass, copper containing respiratory proteins made up of polypeptide chains with M_r 70 000–95 000. The complexity of the molecule varies with the species but it is generally accepted that the more complex structures arose by successive dimerisations of a minimal structural unit, often designated as hexamer (1 × 6 mer), constituted of 6 polypeptide chains.

In the scorpion *Androctonus australis*, 8 subunits [1], with different N-terminal amino acid sequences [2] constitute the 34 S (4 × 6 mer) native Hc. The number of copies of each chain being known [3], the next step in the determination of the quaternary structure is the intramolecular location of each subunit. This paper reports how the immunological recognition of the subunits in the native molecule was used to approach the problem of their intramolecular location.

2. Materials and methods

2.1. *Hemocyanin*

Crude native Hc was prepared as in [4] and purified by gel filtration on Biogel A-5m using a 50 mM Tris–HCl buffer (pH 7.5), 10 mM $CaCl_2$. A complete dissociation of the purified Hc was achieved by a 24 h dialysis against a 50 mM Tris–HCl buffer (pH 8.9), 10 mM EDTA. This procedure yielded a mixture of polypeptide chains called 2, 3A, 3B, 4, 5A, and 6, according to the nomenclature in [1], and a dimeric subunit, called fraction 1, built from 2 additional polypeptide chains (3C,5B). This mixture of dissociation products is further designated as subunit mixture.

2.2. *Immunology*

Rabbit antisera were raised against each of the 8 isolated polypeptide chains. The minute quantities of unexpected antibodies were removed by incubation of the antisera with the subunit mixture as in [1].

Line immunoelectrophoreses were done as in [5] using a 0.12 M barbital–barbital Na-buffer (pH 8.6).

3. Results and discussion

3.1. *Immunoprecipitation of native Hc and subunit mixture by antisera specific for a single subunit*

The aim of this experiment was to compare the precipitation of each subunit by its specific antiserum, in the free state and in native Hc. It was expected that the antigenic determinants of external subunits would be easily accessible to antibodies, and therefore would easily precipitate, but that the subunits hidden inside the whole molecule would lead to a weak precipitation.

Two samples, 1 of subunit mixture and 1 of native Hc, were prepared. Their total protein concentrations were identical but the buffer solutions were slightly different. Native Hc was in a 50 mM Tris–HCl buffer (pH 7.5), 10 mM $CaCl_2$ whilst, prior to immunoelectrophoresis, the subunit mixture was dialyzed overnight against the same buffer without $CaCl_2$. This little difference in the buffer solutions was imposed by the necessity to prevent the reassembly of the subunits and by the impossibility of removing calcium from native Hc without risking a dissociation during electrophoresis.

Eight line immunoelectrophoreses were carried out according to the template of fig.1 using the 8 subunit specific antisera.

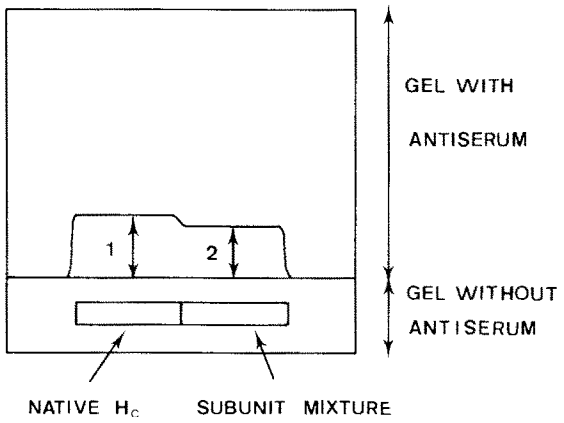

Fig.1. Template for the line-immunoelectrophoreses. The numbers 1 and 2 refer to the migration distances of native Hc and subunit mixture as in tables 1,2.

As shown in table 1, the anti 3A, 3B, 3C, and 5B sera led to a much weaker precipitation when the corresponding subunits were included in native Hc than when they were free. On the contrary, subunits 2, 4, 5A, and 6 precipitated equally well in native Hc as in the free state. This experiment was repeated using crude antisera raised against the isolated subunits. The results were similar to those obtained with the purified antisera, demonstrating that the removal of the unwanted antibodies from the antisera was not responsible for the difference in the migration distances shown in table 1.

The data of table 1 therefore suggest that subunits 2, 4, 5A, and 6 occupy external positions while subunits 3A, 3B, 3C, and 5B have their antigenic determinants hidden inside the Hc molecule. With the anti-subunit 6 serum, the ratio of the migration distance of the lines was even significantly lower than 1, suggesting that the 4 copies of subunit 6 occupy remote external positions, i.e., 1 copy at each corner of the whole molecule.

3.2. *Immunoprecipitation of native Hc and subunit mixture by antisera mixtures*

A careful examination of table 1 shows however that the subunits with a high ratio are present in 2 copies and that subunit with a low ratio occur in 4 copies in native Hc. The question therefore arises whether the intramolecular location or the number of copies is responsible for the observed differences in the position of the precipitation lines.

In order to discriminate between these possibilities, we repeated the experiment of table 1 using binary mixtures of antisera corresponding to the subunits present in only 2 copies in Hc. The amount of each serum added was the same as in the experiment of table 1, so that the total antiserum concentration in the gel was twice higher than in the first experiment. All the antisera mixtures were thus able, as well as the anti 2, 4, 5A, and 6 sera, to react with 4 subunits out of 24 in the whole native molecule. With the subunit mixture two precipitation lines were expected, and indeed observed, except in the case of the mixture of

Table 1
Precipitation of native hemocyanin and subunit mixture in line immunoelectrophoresis using specific antisera for a single subunit

Antisera	Distance in mm[a]		(1)/(2) ratio	No. copies of the corresponding subunit in native H[c]
	Native Hc (1)	Subunit mixture (2)		
Anti 2	14	15	0.9	4
Anti 3A	35	3	11.6	2
Anti 3B	42	2.5	16.8	2
Anti 3C	51	5	10.2	2[b]
Anti 4	16	9	1.7	4
Anti 5A	14	10	1.4	4
Anti 5B	50	3	16.6	2[b]
Anti 6	10	16	0.6	4

[a] Distance between the trough and the precipitation line
[b] Present in subunit mixture as dimeric subunit '1'
[c] Determined by 3 independent methods (see [3])

Table 2
Precipitation of native hemocyanin and subunit mixture in line immunoelectrophoresis using binary mixtures of antisera

Antisera mixture	Distance in mm		(1)/(2) ratio
	Native Hc (1)	Subunit mixture[a] (2)	
Anti 3A + anti 3B	17	4	4.2
Anti 3A + anti 3C	37	6	6.2
Anti 3A + anti 5B	15	4.5	3.3
Anti 3B + anti 3C	31	4.5	6.9
Anti 3B + anti 5B	33	4.5	7.3
Anti 3C + anti 5B	36	3.5	10.2

[a] In order to minimize the risk of misinterpretation, only the highest precipitation line has been taken into account

anti 3C + 5B sera because the corresponding subunits constitute a stable dimer (fraction 1). The results are shown in table 2.

The comparison with table 1 clearly shows that in all cases the addition of the second antiserum in the gel decreased the distance between the trough and the line of native Hc, and therefore facilitated the precipitation. However, the ratios of the migration distances of the native Hc line to the subunit mixture line were still 2–9-times higher than with the anti 2, 4, 5A, and 6 sera.

3.3. *Possible occurrence of soluble immunocomplexes*

To a certain extent, the weak precipitation of the Hc by the antisera raised against subunits present in only 2 copies could be explained by the occurrence of soluble immunocomplexes. Such complexes could result from the presence in native Hc, of too few (1 or 2) or grouped antigenic determinants leading to steric limitations hindering the formation of the 3-dimensional network. In order to detect the presence of soluble complexes, native Hc was incubated with each of the anti 3A, 3B, 3C, and 5B sera for 60 min at 37°C in the 50 mM Tris–HCl buffer (pH 7.5), 10 mM $CaCl_2$. After fractionation of the reaction mixtures by thin-layer gel chromatography on Sephadex G-200 SF, the excluded fraction was scraped, and eluted as in [6]. Then the purified excluded fractions were examined by electron microscopy. No structure typical of soluble immunocomplexes such as dimer or 1-dimension polymer of native Hc was observed.

4. Conclusion

This paper reports that the subunits 3A, 3B, 3C, and 5B from *Androctonus australis* Hc are not easily accessible to antibodies in the native molecule whereas in subunits 2, 4, 5A, and 6, the bulk of antigenic determinants react readily with antibodies. These data show that the subunits present in 2 copies in native Hc (3A, 3B, 3C, and 5B) are located inside the molecule whilst the subunits present in 4 copies (2, 4, 5A, and 6) occupy external positions.

Acknowledgements

We thank Mrs S. Compin for her technical assistance and are indebted to Professor G. Préaux for critical discussion of the manuscript.

References

[1] Lamy, J., Lamy, J. and Weill, J. (1979) Arch. Biochem. Biophys. 193, 140–149.
[2] Jollès, J., Jollès, P., Lamy, J. and Lamy, J. (1979) FEBS Lett. 106, 289–291.
[3] Lamy, J., Lamy, J., Sizaret, P. Y. and Weill, J. (1980) in: Invertebrate Oxygen Binding Proteins: Structure, Active Site and Function (Lamy, J. ed) Dekker, New York, in press.
[4] Lamy, J., Lamy, J., Baglin, M. C. and Weill, J. (1977) in: Structure and Function of Haemocyanin (Bannister, J. V. ed) pp. 37–49, Springer-Verlag, Berlin.
[5] Axelsen, N. H., Kroll, J. and Weeke, B. (1973) A Manual of Quantitative Immunoelectrophoresis. Methods and Applications, Scand. J. Immunol., vol. 2, suppl. 1.
[6] Lamy, J., Lamy, J., Sizaret, P. Y., Maillet, M. and Weill, J. (1977) J. Mol. Biol. 118, 869–875.