Journal of Chromatography, 195 (1980) 277–280
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,785

Note

Human oxymyoglobin: isolation and characterization

TOMOHIKO SUZUKI, YOSHIAKI SUGAWARA, YUKIO SATOH and KEIJI SHIKAMA*
Biological Institute, Tohoku University, Sendai (Japan)
(First received January 22nd, 1980; revised manuscript received February 26th, 1980)

In red muscles such as the cardiac and the skeletal, myoglobin plays an essential role in maintaining aerobic metabolism, both as an oxygen store and by facilitating oxygen diffusion[1–3]. When these muscles suffer from ischemia or other injuries causing cell destruction, the soluble myoglobin will be cleared into blood with myoglobinemia and even into urine with myoglobinuria. The very sensitive quantitation of myoglobin in serum and urine can therefore provide an important new diagnostic test for acute myocardial infarction as well as for other muscular diseases, such as crush syndrome, progressive muscular dystrophy and polymyositis.

In response to the recent increasing need for highly purified human myoglobin for radioimmunoassay[4] and enzyme immunoassay[5], this communication deals with the isolation and characterization of oxymyoglobin from human muscle.

In contrast to the classical preparations of myoglobin in the met-form[6–10], modern procedures for isolating oxymyoglobin directly from muscle tissues all stem from the method of Shikama and co-workers[11]. This has been improved with some refinements and controls using bovine heart muscle, a more readily available source[12–16].

In work described in this report, essentially the same procedure was successfully applied for isolating oxymyoglobin from human muscle for the first time.

EXPERIMENTAL

Myoglobin was extracted overnight at pH 8.0 from the minced, partially thawed muscle (1 kg) of human psoas with 1.5 volumes of cold distilled water. The muscle was obtained at autopsy from adult patients who died of non-muscular disorders and was stored at −5°C by the First Department of Internal Medicine, Tohoku University School of Medicine, Sendai. All procedures were carried out at low temperature (0–4°C) as far as possible. The insoluble material was removed by centrifugation at 3000 *g* for 10 min, and the supernatant was decanted through a doubled gauze cloth to remove fatty substances. This extract was then fractionated with ammonium sulphate between 60 and 100% saturation at pH 7.0 in the presence of $5 \cdot 10^{-4}$ *M* EDTA. The precipitate was centrifuged at 30,000 *g* for 15 min and dissolved in a minimum volume of 5 m*M* Tris–HCl buffer (pH 8.4). The solution was then dialyzed against the same buffer containing $5 \cdot 10^{-4}$ *M* EDTA. The crude myoglobin solution (*ca.* 500 ml), which still contained a large amount of hemoglobin, was applied to four Sephadex G-50 columns (Pharmacia, Uppsala, Sweden; fine, 90 × 5 cm I.D.) equil-

0021-9673/80/0000–0000/$02.25

ibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The column was eluted with the same buffer to separate myoglobin completely from hemoglobin, and the effluent myoglobin solution (*ca.* 500 ml) was dialyzed against 5 m*M* Tris–HCl buffer pH 8.4 containing $5 \cdot 10^{-4}$ *M* EDTA. At this stage about 60% of the myoglobin was in the oxy-form.

The dialyzed myoglobin solution was applied to a DEAE–cellulose column (Whatman DE-32, 15 × 4 cm I.D.), which had been equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The column was washed with a large volume of 15 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 60 ml/h, until the major brown band of metmyoglobin was eluted completely. The major oxymyoglobin component was then eluted with 30 m*M* Tris–HCl buffer (pH 8.0). When concentration was required, the effluent oxymyoglobin solution was dialyzed and applied to a short DEAE–cellulose column (2 × 4 cm I.D.) which had been equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The myoglobin was then eluted with 50 m*M* Tris–HCl buffer (pH 8.0). The yield of the native oxymyoglobin was *ca.* 0.54 g from 1 kg of human psoas muscle, whose total myoglobin content was *ca.* 0.9 g or 0.056 mmole per kg of wet weight.

RESULTS AND DISCUSSION

In this procedure the essential step was the chromatographic separation of oxymyoglobin (MbO_2) from metmyoglobin (metMb) in the hemoglobin-free extract on a DEAE–cellulose column. A typical elution profile of human myoglobin on DEAE–cellulose is shown in Fig. 1. This clearly shows that the absorbance ratio of 582 nm/592 nm, used for identification[12], changed from 1.0 for the first major peak

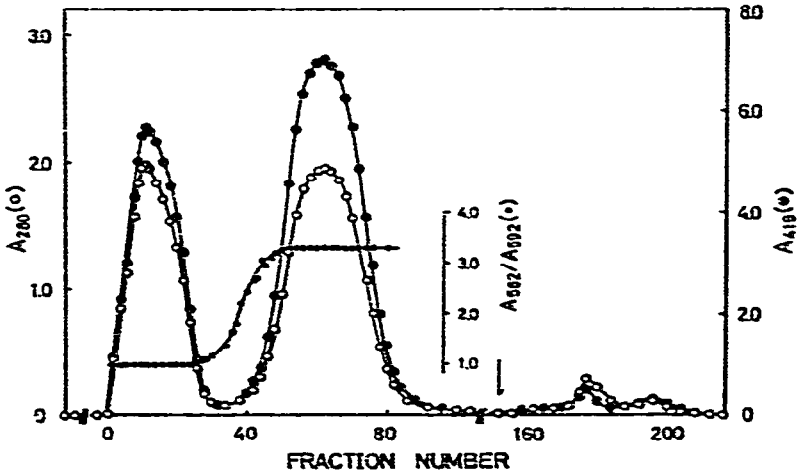

Fig. 1. DEAE–cellulose chromatography of human myoglobin. Myoglobin (50 ml, 270 mg) was applied to a DEAE–cellulose column (20 × 3 cm I.D.), equilibrated with 5 m*M* Tris–HCl buffer (pH 8.4). The major fractions were eluted with 15 m*M* Tris–HCl buffer (pH 8.0) at a flow-rate of 30 ml/h. At the point indicated by an arrow, the buffer was changed to 50 m*M* Tris–HCl (pH 8.0) for elution of the minor components. The protein and the heme protein levels were monitored by the absorbances at 280 nm (○) and at 419 nm (●), respectively. MbO_2 and metMb were identified by the absorbance ratio of 582 nm/592 nm (•). Fraction size: 7 ml.

to 3.3 for the second, indicating the presence of metMb and MbO_2, respectively, with a satisfactory separation. It should be noted that two faint coloured bands remained in the column even after the complete elution of the major fractions. These minor components were readily eluted by changing the buffer to 50 m*M* Tris–HCl (pH 8.0), and were identified as the met-form for the former peak and the oxy-form for the latter. The content of the minor components was less than 8% of the total myoglobin obtained, and no difference was observed in the visible spectrum between the major and the minor.

Heterogeneity in human metmyoglobin preparations has been previously reported. Rossi-Fanelli and Antonini first observed the presence of three components in paper electrophoresis[7]. Perkoff *et al.*[9] found four fractions on DEAE–cellulose chromatography, but only two components were demonstrable in starch gel electrophoresis when the metmyoglobin used was converted into cyanmetmyoglobin. The major component accounted for 75–80% of the total myoglobin, and the remaining minor one(s) appeared to differ in the primary structure. Boesken *et al.*[10] also found three bands in polyacrylamide gel electrophoresis. From the presence of only one band in the crude muscle extract, however, they concluded that alteration in surface charge might occur after purification.

To examine the purity of the major products of our primary concern, therefore, the MbO_2 and metMb were subjected to disc electrophoresis on 8% polyacrylamide gel in 0.3 *M* Tris–HCl buffer (pH 8.9) as well as in 0.1% SDS plus 0.1 *M* Tris–Bicine buffer (pH 8.3). They showed a single band in both gels.

The spectroscopic properties of human major MbO_2 are compared in Table I with those of native MbO_2 isolated from other species. For sperm whale myoglobin, the polymorphic forms were first developed on a DEAE–Sephadex column (Pharmacia, A-25) with 15 m*M* Tris–HCl buffer (pH 8.7). The major fraction was then applied to a CM–cellulose column (Whatman, CM-32) equilibrated with 5 m*M* Tris–HCl buffer (pH 7.0) to separate the MbO_2 completely from the metMb with 10 m*M* Tris–HCl buffer (pH 7.5).

TABLE I

ABSORPTION MAXIMA, EXTINCTION COEFFICIENTS AND CHARACTERISTIC EXTINCTION RATIOS OF MAJOR OXYMYOGLOBINS AT pH 8.0*

Source (ref.)	*Absorption maximum (nm) (extinction coefficient ($mM^{-1}\,cm^{-1}$))*				*α/β*	*γ/UV*
	α	*β*	*γ*	*UV*		
Human	582 (15.4)	544 (14.4)	418 (133)	280 (36.9)	1.07	3.60
Horse (11)	582 (15.3)	544 (14.3)	418 (133)	281 (36.3)	1.07	3.66
Bovine (13)	581 (15.5)	544 (14.5)	418 (134)	280 (36.4)	1.07	3.68
Sperm whale	581 (15.4)	543 (14.3)	418 (129)	280 (36.6)	1.08	3.52

* The concentration of myoglobin was determined after conversion into cyanmetmyoglobin using the extinction coefficient of 11.3 $mM^{-1}\,cm^{-1}$ at 540 nm on the basis of mol.wt. 17,000 by Drabkin[17], except that for sperm whale myoglobin the value of 10.7 $mM^{-1}\,cm^{-1}$ obtained by Hanania *et al.* was used[18].

The extinction ratio of α- to β-maximum (α/β) can provide a most sensitive and useful criterion for estimating the extent of contamination of metMb in the preparations of MbO_2. The values of 1.07–1.08 are the highest ratios obtained so far for native MbO_2 preparations. While this drops to less than 1.00, one must recognize that such a preparation contains more than 30% of metMb at pH 7. This is mainly due to the rapid autoxidation of MbO_2 to metMb, the mechanistic details of which have been extensively studied[11,13–16]. Therefore, the rate of autoxidation of human MbO_2 was measured according to our standard procedure[16], and the observed first-order rate constant, k_{obs}, was determined as follows: $0.83 \cdot 10^{-2}\ h^{-1}$ for human MbO_2, $0.72 \cdot 10^{-2}\ h^{-1}$ for bovine, and $0.50 \cdot 10^{-2}\ h^{-1}$ for sperm whale in 0.1 *M* phosphate buffer, pH 7.2 at 25°C. Although human MbO_2 is oxidized more easily to metMb with a half-life period of 83.5 h at pH 7.2 and 25°C, it is sufficiently stable for many purposes if stored and handled at low temperature (0–4°C) as far as possible, because there is a marked effect of temperature on the autoxidation rate[13].

ACKNOWLEDGEMENTS

We are indebted to Dr S. Ebina, Central Research Laboratory, Fukushima Medical College, Fukushima, Japan for his valuable comments on myoglobinemia and myoglobinuria.

REFERENCES

1 H. Theorell, *Biochem. Z.*, 268 (1934) 73–82.
2 G. A. Millikan, *Proc. Roy. Soc., London, Ser. B*, 123 (1937) 218–241.
3 J. B. Wittenberg, *Physiol. Rev.*, 50 (1970) 559–636.
4 M. J. Stone, J. T. Willerson, C. E. Gomez-Sanchez and M. R. Waterman, *J. Clin. Invest.*, 56 (1975) 1334–1339.
5 E. Engvall, K. Jonsson and P. Perlmann, *Biochim. Biophys. Acta*, 251 (1971) 427–434.
6 H. Theorell, *Biochem. Z.*, 252 (1932) 1–7.
7 A. Rossi-Fanelli and E. Antonini, *Arch. Biochem. Biophys.*, 65 (1956) 587–590.
8 W. H. Luginbuhl, *Proc. Soc. Exp. Biol. Med.*, 105 (1960) 504–505.
9 G. T. Perkoff, R. L. Hill, D. M. Brown and F. H. Tyler, *J. Biol. Chem.*, 237 (1962) 2820–2827.
10 W. H. Boesken, S. Boesken and A. Mamier, *Res. Exp. Med.*, 171 (1977) 71–78.
11 I. Yamazaki, K. Yokota and K. Shikama, *J. Biol. Chem.*, 239 (1964) 4151–4153.
12 T. Gotoh, T. Ochiai and K. Shikama, *J. Chromatogr.*, 60 (1971) 260–264.
13 T. Gotoh and K. Shikama, *Arch. Biochem. Biophys.*, 163 (1974) 476–481.
14 T. Gotoh and K. Shikama, *J. Biochem. (Tokyo)*, 80 (1976) 397–399.
15 Y. Sugawara and K. Shikama, *Sci. Rep. Tohoku Univ., Ser. IV (Biol.)*, 37 (1979) 253–262.
16 K. Shikama and Y. Sugawara, *Eur. J. Biochem.*, 91 (1978) 407–413.
17 D. L. Drabkin, *J. Biol. Chem.*, 182 (1950) 317–333.
18 G. I. H. Hanania, A. Yeghiayan and B. F. Cameron, *Biochem. J.*, 98 (1966) 189–192.