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A contaminant in myoglobin preparations: real or artifact?

In contrast to the classical preparations of myoglobin in the met-form, modern procedures for isolating oxymyoglobins (MbO_2) directly from muscle tissues all stem from the method of Yamazaki *et al.* (1964). However, there is a report that some MbO_2 preparations contained a tightly bound contaminant which affected the O_2 - and CO-binding properties (Keyes *et al.*, 1971). This contaminant was characterized by the changes in the spectrum of MbO_2 due to the addition of 0.5 M-KBr to the solution at pH 8.5; the KBr difference spectrum showed a maximum at about 405–410 nm. Although its identity is not yet known, the contaminant has been referred to as K-substance, and was removed by gel filtration on a Sephadex G-25 column in the presence of a strong reducing agent. In this communication, we demonstrate that only the aquo form of metmyoglobin, present as a contaminant in MbO_2 preparations, can form the complex with Br^- to give the characteristic KBr difference spectrum.

Native oxymyoglobins were isolated directly from bovine heart muscle and sperm whale skeletal muscle according to our standard procedures (Shikama & Sugawara, 1978; Suzuki *et al.*, 1980). Our fresh MbO_2 samples showed no spectral changes on the addition of 0.5 M-KBr at any given pH. The samples used had a value of 1.07 for the absorbance ratio of α - to β -maximum (α/β), a most sensitive and useful criterion for estimating the extent of contamination of metmyoglobin in preparations of MbO_2 (Suzuki *et al.*, 1980). When stored MbO_2 samples, with an α/β ratio of less than 1.05, were used, the characteristic difference spectra appeared after the addition of 0.5 M-KBr to the solutions, and the spectra were not changed even after the samples were subjected to gel filtration on a Sephadex G-25 column (40 cm $\times$ 2.5 cm) equilibrated with 1 mM-Tris/HCl buffer, pH 8.4. These spectra were in accord with those characterized by Keyes *et al.* (1971) for a contaminant in sperm whale MbO_2 preparations after the addition of KBr in 1 mM-Tris/sulphate buffer, pH 8.5.

Therefore, difference spectra were measured after the addition of 0.5 M-KBr to a myoglobin solution (10 μM) consisting of fresh MbO_2 and metmyo-

globin in various ratios in 25 mM-Tris/HCl buffer, pH 8.4, against the respective samples of myoglobin without 0.5 M-KBr. As shown in Fig. 1(a), the magnitude of the difference absorption at 410 nm increases with increasing amounts of metmyoglobin in the MbO_2 solutions.

Fig. 1. Difference spectra for myoglobin preparations in the presence and absence of 0.5 M-KBr at 2°C

(a) Myoglobin solutions (10 μM) consisting of MbO_2 and metmyoglobin in the following ratios in 25 mM-Tris/HCl buffer, pH 8.4: (1) fresh MbO_2 ; (2) 10% metmyoglobin and 90% MbO_2 ; (3) 50% metmyoglobin and 50% MbO_2 ; (4) 100% metmyoglobin. (b) Metmyoglobin solutions (10 μM): (1) hydroxide-metmyoglobin in 0.1 M-phosphate buffer, pH 11.8; (2) aquo-metmyoglobin in 0.1 M-acetate buffer, pH 5.4.

Our further examination has revealed that only the aquo and not the hydroxide form of metmyoglobin gives the bromide complex (Fig. 1b). The peak at 410nm in the difference spectrum for the aquo complex has ΔA of $2.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, which appears 30 times larger than this if the reference is taken to the hydroxide form. Other studies (results not shown) have revealed that, in the pH range where metmyoglobin changes from the aquo to the hydroxide species ($pK_a = 8.9$), therefore, the mode and magnitude of the contribution of the contaminant metmyoglobin to the KBr-induced difference spectra are very complicated.

In conclusion, the MbO_2 preparations used by Keyes *et al.* (1971) were estimated to contain almost 10% of metmyoglobin. It is quite probable that such a considerable amount of contaminant metmyoglobin could alter the ligand-binding properties. Their way to remove the contaminant was consequently to reduce it to the ferrous form on gel filtration in the presence of a strong reducing agent (Keyes *et al.*, 1971; Rifkind *et al.*, 1977). Such contamination is mainly due to the rapid autoxidation of MbO_2 to metmyoglobin, for instance, with a half-life of less than 0.5 day at 35°C and pH 7.0. The mechanistic details of this process have recently

been studied in terms of a nucleophilic displacement of O_2^- from MbO_2 by the entering water molecule or hydroxide anion, with the iron resulting in the ferric form (Shikama & Sugawara, 1978; Sugawara & Shikama, 1980; Satoh & Shikama, 1981).

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