STUDIES ON COBALT-RECONSTITUTED TROUT HEMOGLOBINS

Ernesto E. DI IORIO, Evandro FIORETTI, Isilia ARIANI, Franca ASCOLI, Giuseppe ROTILIO and Maurizio BRUNORI

Laboratory of Molecular Biology, University of Camerino, Camerino and *CNR Center of Molecular Biology, Institutes of Chemistry and Biochemistry, Faculty of Medicine, University of Rome, Rome, Italy*

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1. Introduction

Thermodynamic and kinetic studies of the oxygen and carbonmonoxide binding to trout Hb IV, the main hemoglobin component of trout's blood, have recently contributed to the understanding of the molecular mechanism and the physiological role of the Root effect, i.e., the drop in oxygen affinity and cooperativity observed at low pH. This effect has been interpreted in the framework of a classical two states model [1], as a stabilization by protons or organic phosphate of the low affinity form of the ligand-bound derivative [2].

On the other hand, in the last few years reconstitution of myoglobin and hemoglobin containing cobalt in the place of iron have been successfully employed to elucidate structure–function relationships in hemeproteins [3,4]. The reaction of these cobalt-reconstituted hemeproteins with oxygen has been shown to be cooperative and reversible. Moreover, the paramagnetic character of the Co(II) allowed a detailed EPR study of the deoxy- and oxy-state of the reconstituted proteins [4]. The availability of globins from Hb I and Hb IV from trout blood [5] and the purpose of gaining new relevant information on the effect of protons on the liganded and the unliganded derivative of these proteins, prompted to prepare cobalt containing trout hemoglobins and to study their functional and EPR properties.

2. Materials and methods

Cobalt-*meso*-Hb IV and cobalt-*proto*- and *meso*-Hb I were obtained by reconstitution of trout globins I and IV prepared as in [5] with the corresponding cobalt-porphyrins. Cobalt-*meso*-porphyrin was prepared according to [6] and cobalt-*proto*-porphyrin was a kind gift of Professor T. Yonetani. The reconstitution products were prepared under anaerobic conditions, at 4°C, essentially following the method for cobalt-human hemoglobins [3]. Cobalt-Hb I equilibrated in 0.01 M Tris buffer (pH 9.1) was absorbed on a DEAE A-50 column and eluted using a linear gradient of 10 mM Tris–HCl (pH 9.1) – 100 mM Tris–HCl (pH 6.5). Cobalt-*meso*-Hb IV in 0.01 M Tris–HCl (pH 6.5) was eluted from a CM Sephadex C-50 column with a linear gradient of 50–100 mM phosphate buffer (pH 6.5).

The oxygen binding curves were obtained with the automatic system developed [7,8] with minor technical modifications. X-band EPR spectra were recorded on a Varian mod. E4 apparatus at −150°C.

3. Results and discussion

The optical and functional properties of trout cobalt-hemoglobins differ as expected from the results on Hb A [3]. Compared to the latter, the absorption maxima of the deoxygenated trout cobalt-Hb I and IV are blue-shifted by ~1 nm (see table 1), similarly to that reported for the iron-reconstituted trout hemoglobins [9].

The cobalt-substituted human hemoglobins have lower oxygen affinities than the iron derivatives [3]; a similar effect, but much more enhanced, was observed in trout Hb. The oxygen affinities of

Table 1
Spectral data of cobalt-reconstituted deoxy-hemoglobins (0.1 M phosphate buffer (pH 7.0) 20°C)

	λ_{max}[a]	ϵ_{mM}[a]
Proto-Co-Hb I	551 (552)	19 (17)
	401 (402)	117 (110)
Meso-Co-Hb I	541 (542)	18 (15)
	391 (392)	138 (130)
Proto-Co-Hb IV	551 (552)	–
	400 (402)	–
Meso-Co-Hb IV	541 (542)	–
	391 (392)	–

[a] The values in parenthesis refer to the corresponding derivatives of Hb A [3]

cobalt-*proto*-Hb I and cobalt-*meso*-Hb IV are so low that no quantitative oxygenation curves could be obtained. From the spectra in pure oxygen, in air and in the presence of dithionite, a $\log p_{\frac{1}{2}} > 3$ ($p_{\frac{1}{2}} >$ 1000 mm Hg) was estimated for these derivatives at 20°C. The fully oxygenated derivative could be obtained only in the case of cobalt-*meso*-Hb I under pure oxygen (λ_{max} 559, 528 and 428 nm). This protein binds oxygen reversibly, and displays a small but significant cooperativity (n = 1.2). The value of $\log p_{\frac{1}{2}} = 2.4$ at 20°C is in agreement with a lower affinity for oxygen than cobalt-*meso*-Hb A [3]. The oxygenation curves for this reconstituted protein are pH independent, in agreement with data reported on the native Fe-containing protein [2]. This indicates, as expected, that the absence of heterotropic effects characteristic of trout Hb I is independent from the nature of both the metal and the porphyrin. The overall heat of oxygenation (−1.5 kcal/mol), calculated from the oxygen binding curves determined at different temperatures is very low and of the same order of magnitude of that measured for native trout Hb I. The differences in the overall heat of oxygenation observed [3] between iron and cobalt-containing human hemoglobins could not be detected in this case probably because of the very low enthalpy values. Since according to our current interpretation ([10], M. B., M. Coletta, B. Giardina, S. Gill, J. Wyman, unpublished), the very low heat of ligand binding characteristic of trout Hb I may be related to the high enthalpy change associated with the allosteric transition, these new results suggest that also in cobalt-Hb I a similar phenomenon may occur.

EPR spectra of both reconstituted Hbs indicate that at the temperature of the experiment (−150°C) these proteins are fully oxygenated (fig.1) and exhibit the typical free radical signal around $g = 2$, with the same features (axial symmetry and hyperfine structure) already shown for human cobalt-HbO_2 [4]. The characteristic hyperfine structure is however less resolved at higher pH; this effect may, at least in part, be due to smaller hyperfine splitting, particularly evident for cobalt-*meso*-Hb I. Decrease of the hyperfine coupling constant value has been inter-

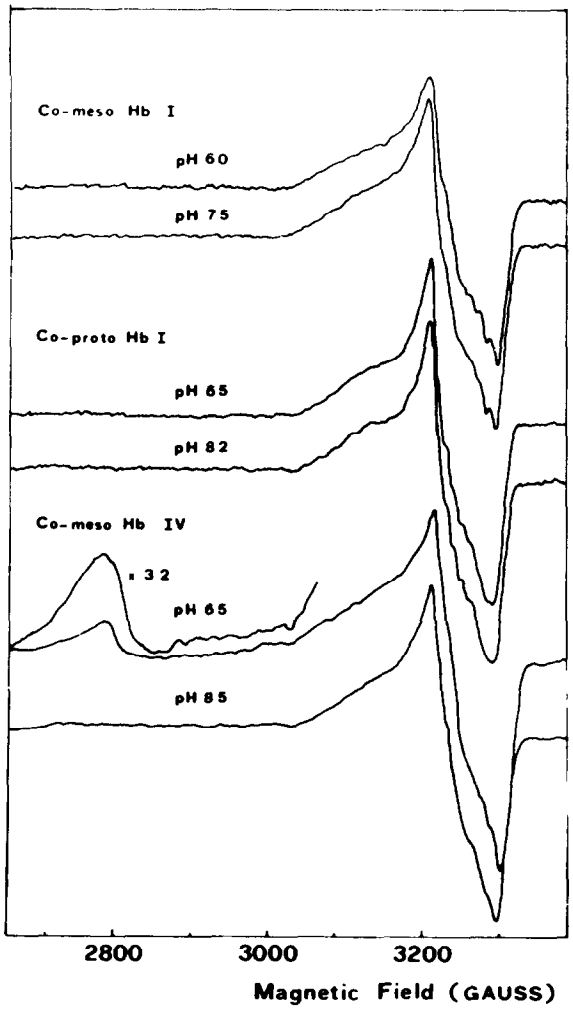

Fig.1. EPR spectra of cobalt-reconstituted Hb, in air: cobalt-*meso*-Hb I (pH 6 and 7.5); cobalt-*proto*-Hb I (pH 6.5 and 8.2); cobalt-*meso*-Hb IV (pH 6.5 and 8.5). Modulation amplitude 5 G; microwave power 20 mW.

preted as an indication of a greater spin density transfer from cobalt to oxygen [11] and has been related to a hydrogen bond formed between oxygen and the distal histidine [4,12]. The EPR spectra of cobalt-*meso*-Hb IV in air (fig.1) indicate that at high pH (8.5) this protein as well is fully oxygenated at −150°C; in contrast at low pH (6.5) a substantial amount of deoxygenated protein is present, as shown by the characteristic EPR absorption ($g_\perp \simeq 2.3$) observed under these conditions.

The EPR spectra of the deoxygenated cobalt-*proto*-Hb I, cobalt-*meso*-Hb I and cobalt-*meso*-Hb IV in the presence of sodium dithionite are shown in fig.2. These derivatives exhibit the usual spectrum with axial symmetry ($g_\perp \simeq 2.3, g_\parallel \simeq 2.03$) and the characteristic hyperfine and superhyperfine structure of the cobalt and proximal histidine nitrogen nuclei [4]. A small but significant change is evident in the $g_\perp$ region of the cobalt-*meso*-Hb IV spectra, between pH 6.5 and 8.5. This change may be ascribed to slightly different geometries of the complex at different pH values.

The more significant findings obtained by EPR may, therefore, be deduced from the spectral properties of Hb IV, both oxy and deoxy, at different pH values. Although it is not possible to quantitate the observation it is striking that even at −150°C, cobalt-*meso*-Hb IV is partially deoxygenated at pH 6.5. This is an indication together with the data obtained on Fe-*proto*-Hb IV [13], of the large forces opposing binding of oxygen to the protein in the *T*-state. In addition, the pH-dependent change at $g \simeq 2.3$ (fig.2) is an indication that there may be a proton-induced structural change(s) even with the deoxygenated derivative of Hb IV in the pH region where the Root effect is operative. This new observation, if substantiated by other spectroscopic techniques, may cast some doubt as to the rigorous applicability of a simple two-state model to the pH behaviour of trout Hb IV, in agreement with a recent analysis of the functional data [10].

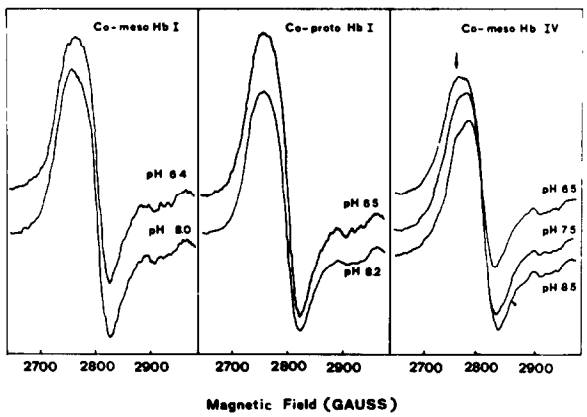

Fig.2. EPR spectra of cobalt-reconstituted Hb in the presence of dithionite: cobalt-*meso*-Hb I (pH 6 and 8); cobalt-*proto*-Hb I (pH 6.5 and 8.2); cobalt-*meso*-Hb IV (pH 6.5, 7.5 and 8.5). EPR parameters as in fig.1. Only the $g_\perp$ region and the first hyperfine line of the $g_\perp$ showing the typical superhyperfine triplet of nitrogen, is presented in the figure.

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