The Orientation of the Electric Field Gradient Tensor in CO-Liganded Myoglobin

F. Parak, U. F. Thomanek, D. Bade, and B. Wintergerst

Physik-Department der Technischen Universität München, Garching

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Myoglobin-CO Single Crystal, Mössbauer Spectra, EFG-Tensor

The EFG-tensor at the position of the Fe-atom of CO-liganded sperm whale myoglobin has been investigated by nuclear γ-resonance absorption experiments on single crystals. In addition the temperature dependence of the quadrupole splitting of the 14.4 keV level of the iron nucleus was measured. An unambiguous solution for the magnitude and the orientation of the field gradient tensor could only be obtained with the assumption that a C_2-axis perpendicular to the haem plane is one principal axis of the electric field gradient tensor. Within this solution the electronic structure of the iron is described by a singlet ground state with $\eta_z = 0.75$ and the largest EFG component perpendicular to the haem plane.

1. Introduction

Recently a phase determination of the $60\bar{3}$-reflection of a CO-liganded myoglobin single crystal (MbCO) has been published[1]. The phase information was obtained from the interference pattern of γ-resonance scattering at the ^{57}Fe nuclei and the Rayleigh scattering at the electrons. The nuclear transition of ^{57}Fe is split into two resonance lines by the electric hyperfine interaction. Therefore, the analysis of the interference pattern involves two nuclear scattering amplitudes with a magnitude and a polarization depending on the orientation of the incoming and the scattered γ-ray with respect to the electric field gradient tensor (EFG-tensor) at the position of the ^{57}Fe nuclei. Since the orientation of the EFG was not known we used in ref. 1 the following approach: We measured the nuclear γ-resonance absorption of the MbCO crystal, still oriented in the position of the $60\bar{3}$-reflection. The relative intensities of the quadrupole lines gave us the relative magnitudes of the scattering amplitudes. This approximation is valid only for small scattering angles. In this paper we want to describe experiments yielding the orientation of the EFG. This will enable us in the future to perform a more rigorous analysis of phase experiments on MbCO.

Moreover, the determination of the electronic level scheme of the Fe in MbCO is of large interest for the understanding of the binding properties of myoglobin. Of course, an electronic level scheme cannot completely explain the dynamic binding properties of the enzyme. Additional investigations by other methods are necessary[2]. However, the electronic level scheme of the iron will always yield highly useful information about the static limit of the MbCO-complex. As was shown in the case of deoxygenated myoglobin, the determination of the EFG-tensor[3] largely influences the picture of the electronic term scheme[4–7]. The present data of the EFG-tensor in MbCO should also give a clearer insight into the electronic structure of the Fe-atom at the active center of this protein.

2. Materials and Methods

In order to obtain the orientation of the EFG we performed two series of experiments. First the nuclear γ-resonance absorption spectrum was measured on single crystals of MbCO. To obtain these crystals, we first prepared ^{57}Fe enriched sperm whale metmyoglobin according to reference[8]. Ammoniumsulfate was added to a 3 mg/ml metmyoglobin solution until it became 2.5 M. The pH-value was stabilized to 7.0 by ammoniumphosphate. The solution was degassed and saturated with N_2. The reduction was performed by $Na_2S_2O_4$ under strict anaerobic conditions. Then the N_2 was exchanged by CO. The MbCO solution was filled into small glass tubes in portions of 0.7 ml. After adding an amount of 80 ... 90 mg of solid ammoniumsulfate each tube was capped under CO atmosphere. The crystallisation occurred in the refrigerator during two days to 10 days.

In ref. 9 a method is described to avoid the distortion of the crystal lattice by expansion of the crystal water during the phase transition from water to ice. Instead of freezing the MbCO crystals this way we simply put them into liquid propane at a

Requests for reprints should be sent to Dr. F. Parak, Physik-Department E 15, Technische Universität München, James-Franck-Str., *D-8046 Garching*.

temperature of about 90 K. The boiling point of propane is 231 K. The heat content of the small protein crystal is rather low and no boiling occurs during the freezing procedure. Therefore, the freezing time becomes considerably shorter than in liquid nitrogen. The frozen crystals were oriented on a X-ray precession camera. The cooling equipment of this camera is described in ref. 9. The crystallographic $b = b^*$-axis of the monoclinic crystal coincided with the K-circle of the precession camera. No distortion of the crystal lattice could be seen on the X-ray film. The crystals were then put into a small N_2-vapor-cryostat, which was mounted on a Siemens 3-circle diffractometer. More details of the equipment will be described elsewhere. A $\vartheta/2\,\vartheta$ scan with MoK_α-radiation yielded the orientation of the reciprocal lattice of the crystal with respect to the experimental equipment. The vapor cryostat together with the crystal could be rotated around the crystallographic b-axis by the angle φ, thus giving different orientations of the EFG-tensor to the incoming γ-ray beam. We used a 220 mCi $^{57}\text{CO}\underline{\text{Rh}}$ source with an effective active area of $1 \times 2.5\ \text{mm}^2$. The data were collected with a Si(Li) detector of an active area of $7 \times 2.5\ \text{mm}^2$ and 2 mm thickness. The Mössbauer spectrometer consists of an electromagnetic driving system according to Kalvius [10] with constant acceleration and a Hewlett Packard multichannel analyser. Only 100 channels were used since the counting rate was rather low.

In addition to the single crystal experiments we have measured the quadrupole splitting of a MbCO absorber at different temperatures. The sample preparation started with a large number of small ^{57}Fe enriched metmyoglobin crystals. These crystals were reduced by sodium dithionite, which was dissolved in 3.8 M ammoniumsulfate. To protect the crystals we started with pure ammoniumsulfate solution, and replaced it after 10 min by a solution with 0.0125 M dithionite. We then increased the dithionite content in 4 steps till we came to a 0.1 M solution. The crystals were then put into a 3.8 M ammoniumsulfate solution, which was saturated with CO gas. This solution was exchanged 3 times. The wet crystals were then filled into a sample holder and frozen under high pressure [9]. The investigations were carried out in a vapor cryostat and a liquid He cryostat. The temperature control stabilized the sample temperature within ± 2 K. A conventional Mössbauer spectrometer has been used.

3. Experimental Results

Fig. 1 shows a Mössbauer spectrum of a MbCO crystal. As fit parameter we obtained: the quadrupole splitting $e^2\,q\,Q/2 = 0.36$ mm/s, the isomer shift $S = 0.19$ mm/s, the experimental linewidth $\Gamma_{\text{exp}} = 0.48$ mm/s, which was assumed to be equal for both lines and $I_+/(I_+ + I_-) = 0.53$ which represents the percentage area of the absorption line at positive velocities. The aim of the experiment was the determination of this ratio for several orientations of the incoming γ-beam with respect to the crystal. For our description we chose a right hand coordinate systems c', a, b, which is fixed to the single crystal. The direction of $\boldsymbol{a}$ and $\boldsymbol{b}$ coincides with the crystallographic a and b axis of the monoclinic MbCO crystal. $\boldsymbol{c}'$ is perpendicular to $\boldsymbol{a}$ and $\boldsymbol{b}$. The direc-

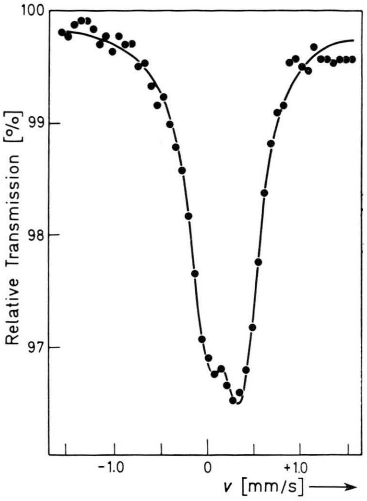

Fig. 1. Mössbauer absorption spectrum of a single crystal of CO-liganded myoglobin. The incident γ-beam was perpendicular to the crystallographic b-axis.

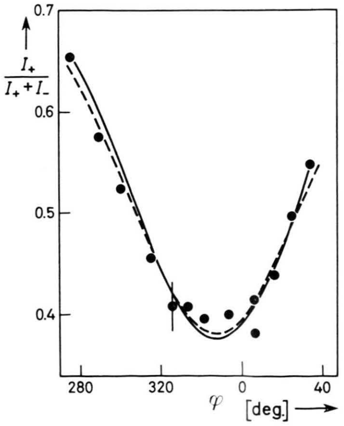

Fig. 2. Experimental angular dependence of the intensity ratio of the two quadrupole lines of a CO-liganded myoglobin single crystal. The solid and the dashed lines are obtained from least squares fits described in the text.

tion of the $\boldsymbol{k}$-vector of the γ-beam in this system is given by $\boldsymbol{k}$ $(\sin\delta\cos\varphi, \sin\delta\sin\varphi, \cos\delta)$. All experiments were performed with $\delta = 90°$. Fig. 2 shows the ratio $I_+/(I_+ + I_-)$ as a function of φ. The solid and the dashed lines are obtained by least squares fits as described later.

Im Fig. 3 we show the temperature dependence of the quadrupole splitting from measurements with the MbCO absorber described before.

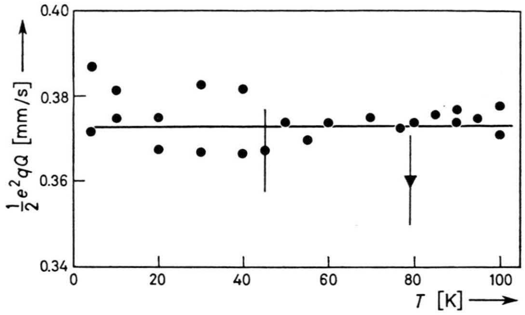

Fig. 3. Temperature dependence of the quadrupole splitting of CO-liganded myoglobin. Full circles: experiments on a polycrystalline absorber. Triangle: average value over a large number of measurements on a single crystal absorber. The solid line is calculated from the electronic level scheme given in the text.

4. Discussion

We have analyzed the single crystal data in two different ways. The first way follows the lines of [5]. MbCO crystallizes in a monoclinic lattice with $a = 65.6$ Å, $b = 31.1$ Å, $c = 34.8$ Å and the monoclinic angle $\beta = 105.5°$ between $\boldsymbol{a}$ and $\boldsymbol{c}$. The crystal contains two molecules of myoglobin in the unit cell. We want to discuss our results in a coordinate system x_i', y_i', z_i', which is fixed to the haem plane of the molecule. The index i characterizes the molecule 1 or 2, respectively.

x' is defined by a straight line from the nitrogen atom NPL 417 to the nitrogen atom NVR 415 of the haem plane and y' by the line from the nitrogen atom NVL 416 to NPR 418 (compare [11]), z' is perpendicular to x' and y'. The system x_1, y_1, z_1 and x_2, y_2, z_2 is obtained from our principal system c', b, a by the Euler transformation with $\alpha_1 = 72.16$, $\beta_1 = 68.85$, $\gamma_1 = 47.57$ and $\alpha_2 = 252.16$, $\beta_2 = 68.85$, $\gamma_2 = 47{,}57$, respectively. For the present purpose the translations may be neglected. As our experiments were performed with $\delta = 90°$, both molecules become indistinguishable and the indices can be omitted. The quadrupole interaction of the EFG with the 14.4 keV level ($I = 3/2$) of ^{57}Fe is described in the principal axis system x, y, z. It is obtained from the $x'\,y'\,z'$ system by the Euler-transformation with the angles χ_1, χ_2, χ_3. To begin with we restrict our discussions to the case that z' and z coincide which means $\chi_1 = \chi$, $\chi_2 = \chi_3 = 0$. This enables us to compare our single crystal experiments directly with the temperature dependence of the quadrupole splitting.

The intensity ratio $I_+/(I_+ + I_-)$ is given by

$$\frac{I_+}{I_+ + I_-} = \frac{3}{8}(1+\cos^2\Theta) - \frac{1}{4}\sin^2\mu\,(3\cos^2\Theta - 1) + \frac{1}{8}\sqrt{3}\,\sin 2\mu\,\sin^2\Theta\,\cos 2\Phi\,. \tag{1}$$

The angles Θ and Φ define the γ-ray direction $\boldsymbol{k}$ in the principal axes system $(x\,y\,z)$. $\boldsymbol{k}$ $(\sin\Theta\cos\Phi, \sin\Theta\sin\Phi, \cos\Theta)$ is obtained from $\boldsymbol{k}$ $(\sin\delta\cos\varphi, \sin\delta\sin\varphi, \cos\delta)$ by the Euler-transformations, described beforehand. The quadrupole interaction Hamiltonian splits the $I = 3/2$ level of ^{57}Fe into two Kramers doublets with the eigenfunctions

$$\begin{aligned} |+\rangle &= \cos\mu\,|\pm\tfrac{3}{2}\rangle + \sin\mu\,|\mp\tfrac{1}{2}\rangle \\ |-\rangle &= -\sin\mu\,|\pm\tfrac{3}{2}\rangle + \cos\mu\,|\mp\tfrac{1}{2}\rangle \end{aligned} \tag{2}$$

and the energy levels

$$E_\pm = \pm 3\,p\,\sqrt{v_{zz}^2 + \frac{1}{3}(v_{xx} - v_{yy})^2}\,. \tag{3}$$

p equals $2.08\,\alpha^2$ if $E_\pm$ is given in mm/s and $\alpha^2 = 0.89$ is the covalency factor [5]. We add the definition

$$\operatorname{tg} 2\mu = \eta_z/\sqrt{3} \tag{4}$$

with

$$\eta_z = (v_{xx} - v_{yy})/v_{zz}\,. \tag{5}$$

η_x and η_y are obtained by cyclic permutation of the indices. Since we have assumed that z and z' coincide, v_{zz} is not necessarily the main component of the EFG-tensor. In contrast to [5] we don't assume $v_{xx} > v_{yy}$. Therefore, the parameter μ is restricted to $0 \leqq \mu \leqq \pi$. For all further details compare [5].

The solid line of Fig. 2 shows the least squares fit of Eqn (1) to the experimental data. As the result we obtain $\eta_z = 0.75$ and $\chi = -4.5°$. In contrast to deoxygenated myoglobin where the main component of the EFG-tensor points into the x-direction[3] we obtain here the main component in the z-direction (C_{4v}-axis). According to[12] the main component of the EFG is assumed to be positive.

We now want to analyse our data in a more general way. According to Zimmermann[13] the unique determination of the EFG-tensor is not possible from Mössbauer spectra of a single crystal, if this crystal has monoclinic symmetry and two molecules in the unit cell. Unfortunately, this is exactly the case of a sperm whale myoglobin crystal. Here it is only possible to determine an effective EFG-tensor, which can be decomposed into the two local EFG-tensors of the two iron atoms of the unit cell in many different ways. From[13] we take the theoretical function

$$\frac{I_+}{I_+ + I_-} = A + B\cos(2\varphi - 2\varphi_0) \qquad (6)$$

which was fitted by a least squares procedure to the experimental data yielding A, B and φ_0 (dashed curve in Fig. 2). The tensor components I_{pq} which are proportional to the EFG-tensor components are obtained from these values using the following relations

$$\begin{aligned} V_{c'c'} &\propto I_{c'c'} = A + B\cos 2\varphi_0 - \frac{1}{2} \\ V_{aa} &\propto I_{aa} = A - B\cos 2\varphi_0 - \frac{1}{2} \qquad (7) \\ V_{c'a} &\propto I_{c'a} = B\cos\left(\frac{1}{2}\pi - 2\varphi_0\right). \end{aligned}$$

The effective EFG-tensor is here described in the coordinate system which is fixed to the single crystal. The components of EFG-tensors on the places of the iron atom 1 and 2 which give together the effective EFG-tensor, have to fulfill the following relations

$$\begin{aligned} V_{c'c'}{}^{(Fe1)} &= V_{c'c'}{}^{(Fe2)}; & V_{c'a}{}^{(Fe1)} &= V_{c'a}{}^{(Fe2)}; \\ V_{aa}{}^{(Fe1)} &= V_{aa}{}^{(Fe2)}; & V_{c'b}{}^{(Fe1)} &= -V_{c'b}{}^{(Fe2)}; \quad (8) \\ V_{bb}{}^{(Fe1)} &= V_{bb}{}^{(Fe2)}; & V_{ab}{}^{(Fe1)} &= -V_{ab}{}^{(Fe2)}. \end{aligned}$$

From the components of the effective EFG-tensor obtained by Eqn (7) one can determine only the components $V_{c'c'}{}^{(Fe1)}$, $V_{bb}{}^{(Fe1)}$, $V_{aa}{}^{(Fe1)}$, and $V_{c'a}{}^{(Fe1)}$ of the local EFG-tensor, whereas the components $V_{c'b}{}^{(Fe1)}$ and $V_{ab}{}^{(Fe1)}$ remain undetermined. The manyfold of the solutions can be described by the parameter Ψ defined by

$$\Psi = \arctan\left(I_{ab}{}^{(Fe1)}/I_{c'b}{}^{(Fe1)}\right). \qquad (9)$$

To simplify the discussion, the local EFG-tensor should be described in a system x, y, z, where it is diagonal. This system is obtained from the $c'ab$-system by an Euler-transformation with the Euler-angles α, β and γ. Fig. 4 shows these Euler-angles as a function of the parameter Ψ together with the local assymetry parameter η_z. Only the range $0 \leqq \Psi \leqq \frac{1}{2}\pi$ corresponding to $0 \leqq \alpha, \beta, \gamma \leqq \pi$ is shown. The solution of the other quadrants can be obtained by Eqn (22) of ref. 13.

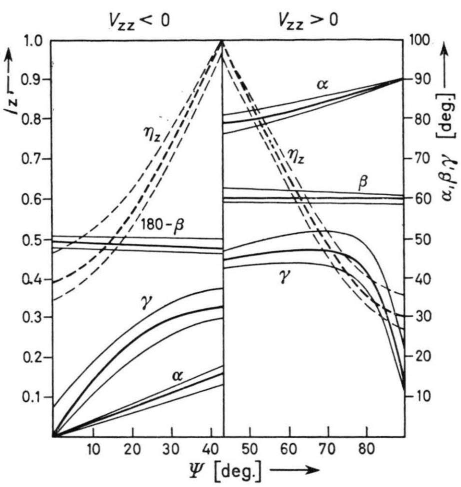

Fig. 4. Correlation between the "effective" EFG-tensor and the EFG-tensors at the two irons within the unit cell of a CO-liganded myoglobin single crystal. The parameter Ψ describes the manyfold of solutions. Each Ψ-value corresponds to one local η_z-value and one set of Euler-angles α, β, γ. Error bars for α, β, γ and η_z are given by the thin lines above and below the central lines. For details see the text.

To compare these results with our previous discussions we limit ourselves to a positive main component of the EFG[12] and to $0 \leqq \Psi \leqq 90°$. Table I gives the orientation of the local EFG-tensor system x, y, z with respect to the system x', y', z', which is fixed to the haem plane for three Ψ-values.

We first discuss the solution of $\eta_z = 0.76$, $\Psi = 55°$. Here the z-axis of the xyz-system is turned by about 12° away from the z'-axis within the $y'z'$-plane. This should be compared with our first result, where z and z' are parallel by definition. There η_z was 0.75 and the angle between x and x' was 4.5°.

The maximum value $\eta_z = 1$ is obtained for $\Psi = 44°$. The orientation of xyz-system in this case remains practically the same as for $\Psi = 55°$. The smallest η_z-value which still yields a positive EFG-tensor is obtained for $\Psi = 90°$ ($\eta_z = 0.30$). In this

Table I. Orientation of the EFG-tensor system $x\,y\,z$ with respect to the hemin system $x'\,y'\,z$. For $0 \leqq \Psi \leqq 90°$ only Ψ values between 44° and 90° yield a positive main component of the EFG-tensor.

Ψ	η_z	α	β	γ	p	$\cos(x, p)$	$\cos(y, p)$	$\cos(z, p)$
44	0.99	78.7	60.7	45.3	x'	1.000	−0.007	−0.023
					y'	0.011	0.985	0.173
					z'	0.021	−0.173	0.985
55	0.76	81.2	60.3	46.8	x'	0.999	−0.054	−0.002
					y'	0.053	0.977	0.204
					z'	−0.009	−0.204	0.979
90	0.30	90.0	59.8	14.1	x'	0.904	0.420	0.079
					y'	−0.423	0.853	0.306
					z'	0.061	−0.310	0.949

case the z-axis is turned about 18° away from the z'-axis.

From these results one may conclude that all solutions within $0 \leqq \Psi \leqq 90°$ yielding a positive EFG-tensor are diagonal in a $x\,y\,z$-system with a z-axis only slightly tilted against the z'-axis of the hemin system. This seems to prove the influence of the nearest neighbours of the Fe-atom to the orientation of the EFG-tensor. If one includes $90° \leqq \Psi \leqq 360°$ the correlation between the ligand symmetry and the orientation of the EFG-tensor vanishes.

There remains the discussion of the temperature dependence of the quadrupole splitting $e^2 q Q/2$. In the temperature range between 4.2 K and 100 K the quadrupole splitting remains practically constant. Similar to CO liganded hemoglobin MbCO should be diamagnetic[14]. Although we know that in the MbCO-complex the d_π and d_{z^2} orbitals of the iron are not metal orbitals as pure as in deoxygenated myoglobin[15], we want to discuss the electronic structure of the iron in MbCO within the crystal field model too. One could then understand the temperature independence of the quadrupole splitting by an isolated $^1A_1$-term as the ground state with $\eta_z = 0$. Then the quadrupole splitting is mainly caused by the direct "lattice" contribution of the nearest neighbours. If one accepts the value $\eta_z = 0.75$, which can be obtained from the single crystal experiment, one has to take into account an influence of the ^3E or the ^5E term. Since the ^5E cannot be directly mixed into the $^1A_1$ term by spin orbit coupling, the ^3E-term has to be the first excited state. Using the theory as described in[5] with $\varkappa = 15.5 \times 10^{-6}$ cm, $\alpha^2 = 0.89$ and $\lambda = 69.0\ \text{cm}^{-1}$ we have calculated a ground state with 80% $^1A_1$ character and 20% ^3E character. Together with the direct contribution governed by the parameter $\varkappa$, an EFG-tensor with $e^2 q Q/2 = 0.373$ mm/s, $\eta_z = 0.75$, and $V_{zz} > 0$ arises from this singlet. As the excited singlet states with mainly ^3E character lie about 600 cm^{-1} above the ground state singlet, the magnetic susceptibility is calculated to be less than 10^{-3} cm^3/mol, as expected for a diamagnetic complex.

We want point out here that our value of $\eta = 0.75$ fits to the Mössbauer spectrum at an external magnetic field of 4.7 T[12], although the authors[12] have limited η to less than 0.4. As prove we have calculated the hyperfine spectra for the interaction of an external magnetic field of 4.7 T and the described EFG-tensor with the iron nucleus by a program to be described elsewhere. Fig. 5 shows the calculated spectrum for $\eta = 0.4$, $e^2 q Q/2 = 0.363$ mm/s [curve (a)] which agrees with the spectrum given by ref. 12. It is compared with the spectrum calculated from the term scheme used here, with $\eta = 0.75$ and $e^2 q Q/2 = 0.373$ mm/s [curve (b)]. As the shape of the spectra is mainly determined by the strong magnetic splitting, the variation of η has only a very small influence and

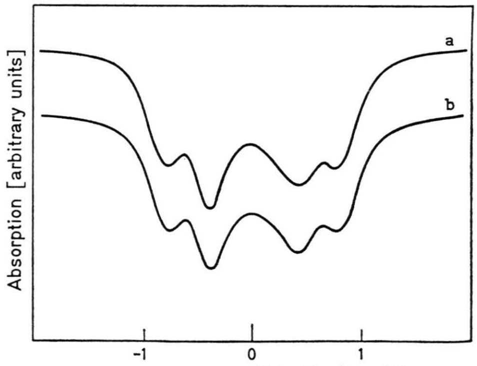

Fig. 5. Calculated Mössbauer absorption spectra of CO-liganded myoglobin at 4.2 K and an externally applied magnetic field of 4.7 T. a) $e^2 q Q/2 = 0.363$ mm/s, $\eta_z = 0.4$; b) $e^2 q Q/2 = 0.373$ mm/s, $\eta_z = 0.75$.

seems to be clearly within the error of the experiment[12].

Finally we want to mention that our value of $\eta = 0.75$ for the ground state EFG-tensor of the iron is correlated with a strong rhombic distortion of the primarily assumed C_{4v} symmetry of the iron environment. This rhombic distortion may be interpreted as an influence of the non-axially bound CO-ligand. In fact it is known from X-ray structure analysis of erythrocruorine[16] and horse hemoglobin[17] that there is a bond angle between the axis of the CO-molecule and the z-axis perpendicular to the haem plane.

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