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Coordination Behavior of O_2 and CO in a Solid Film Consisting of Hemoglobin and Maltose

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Solid hemoglobin (Hb) films were prepared by casting from a mixed solution of human hemoglobin and maltose (wt ratio of Hb/maltose is 60/40). The behavior of O_2 and CO in coordinating to Hb in the films was then observed. The coordination equilibrium constants of both ligands were smaller by about an order of magnitude, and the rate constants (k_{on} and k_{off}) were smaller by about 13 orders of magnitude, than those of aqueous solutions of Hb. Interestingly, O_2 and CO saturation was limited to 52% and 86%, respectively, under a partial pressure of 760 Torr (1 Torr=133.322 Pa) of each gas, in spite of the one-to-one ligand binding ratio in aqueous solutions. This is due to the restraint of the structural change from a tense to a relaxed state in the solid phase. The limited saturation of the coordination in the solid phase was found to increase to 100% when a small amount (5.9%) of water was added; a significant structural change from the tense to the relaxed states followed.

There are many reports on the coordination of ligands, especially O_2 and CO, with Hb,[1—3] and water molecules play an essential role in the coordination behavior by interacting with Hb.[4—15] They form a hydration layer surrounding Hb by hydrogen bonding and also interact directly with heme or proximal histidine in the heme pocket.[12,13] This relates to stabilization of both globin and heme. The removal of the hydration layer induces reversible or irreversible conformational changes[6—11] and causes the oxidation of heme, i.e., metHb formation.[14,15] Only when the conformational change is reversible upon dehydration and rehydration does the dried Hb retain a latent function that can be activated after rehydration.[6,9] The addition of protectors (disaccharides, alcohols, etc.) before dehydration is effective in preserving Hb over long periods of time, with retention of its structure and functions.[16—18] We prepared Hb films by casting the Hb solution with maltose, and no denaturation of Hb was observed.[19]

In this research, we studied the coordination behavior of O_2 and CO with Hb in such a solid film and compared the coordination parameters to those of Hb aqueous solution, relative to the quaternary structural change between the relaxed (R) and tense (T) states. The influences of residual water present on the activation of coordination and on the structural change are also reported.

Experimental

Preparation of Hb Films.[19,20] Human Hb was purified from out-dated red blood cells (Hokkaido Red Cross Blood Center) in accordance with the paper reported previously.[20] A red blood cell solution converted from HbO_2 to HbCO by contact with CO gas was mixed with a 0.2-fold volume of dichloromethane to lyse the red cells and remove stroma. The proteins other than HbCO were denatured by heating the HbCO solution at 60 °C and removed by ultracentrifugation. The resulting HbCO solution was deionized by dialysis against distilled water at 4 °C. HbCO was converted to HbO_2 by irradiation of visible light from a halogen lamp under flowing O_2. The resulting Hb solution was regulated to [Hb]=10 g dl^{-1} and pH 7.2. Each 2 μl of Hb solutions containing 0.3 M (1 M=1 mol dm^{-3}) of maltose was cast on the side wall (1 cm×4 cm) of a sealed quartz cell and dehydrated by dry N_2, O_2, and CO flowing through a silica gel tube into the cells for 2 h at 22 °C. The absence of denaturation of Hb of the resulting films was confirmed by the methods of Evelyn and Malloy[21] and by isoelectric focusing.[22]

Quantitative Analysis of Hb Derivatives. The characteristic absorbances of Hb (430 nm), HbO_2 (415 nm) and HbCO (419 nm) were observed by a UV-visible spectrophotometer (MPS-2000 Shimadzu Co.). The fraction of each derivative was calculated by a calibration curve made using the ratios of the absorbance from the base line between the isosbestic points. After O_2 and CO at various partial pressures were introduced into the quartz cells in which an Hb film was cast, the absorbance spectra of films were measured as a function of time at 22 °C in order to obtain the binding curves.

Sorption Isotherms of O_2. Sorption isotherms of O_2 were measured from the gas pressure by removing the influence of the physical sorption of O_2 onto an Hb film. The absorption amount of O_2 inside the Hb film at a constant volume with a Baraton absolute pressure gauge which was

a part of KA-102 (Makuhari-rikagaku Glass Co.). A deoxyhemoglobin solution was cast on a Teflon plate and allowed to stand in vacuo for 24 h at 22 °C. After the film was mounted on the apparatus, the sample was deoxygenated repeatedly by evacuating for 24 h at 10^{-6} Torr and 22 °C until no change in the volume gauge was observed. The Hb film was subjected to 746 Torr of oxygen pressure at 22 °C for sorption isotherm determination. The initial pressure drop was followed by a further decrease due to the binding of O_2 to the film; finally the pressure became constant due to the equilibrium of gas sorption. The same measurement was carried out for the film of metHb obtained by adding a sufficient amount of $K_3[Fe(CN)_6]$. The physical sorption of the film was extracted from the results.

Quaternary Structural Change. In the aromatic region (274 nm) of the UV spectrum, the films of Hb derivatives were measured to obtain information about their quaternary structures.[23] It was confirmed that the spectra of HbO_2 and HbCO films show good agreement with Hb in the R state in aqueous solutions and are different from that of Hb in the T state. The fraction of the R state was calculated from the ratios of the absorbances at 274 nm and the isosbestic points of Hb and HbO_2.

Water Content of Hb Films. The relative humidity in the quartz cell was controlled to a constant value by flowing gas with the humidity controlled by a conventional method, and the value was monitored with a temperature humidity meter (Palma Co., precision class). Hb fine power obtained from an Hb film was equilibrated in the same manner under controlled humidity. The water content of the powder was measured by TG-DTA (SSC/5200 Seiko Instruments Co.).

Results and Discussion

It was reported that maltose has a great protecting effect on the dehydration of Hb.[24,25] Dehydration without maltose denatures about 50% of Hb to metHb (brown color) and causes many cracks in the surface of a dehydrated Hb film. An HbO_2 film containing 32 wt% maltose is smooth and has a fresh red color like an HbO_2 solution. However, in this case the metHb fraction formed during dehydration was 2% higher than that before dehydration, and 41 wt% maltose was necessary for complete inhibition of metHb formation.[19] The UV-visible absorption spectra of the resulting Hb, HbO_2, and HbCO films almost agreed with those of the corresponding solutions (Table 1). These absorption spectra are distinct from those of anhydroHb as reported by Keilin[9] and Haurowitz.[10] It was reported by them that the sixth coordination bond of the iron atom occupied by a water molecule was replaced by the nitrogenous group of the distal histidine after vacuum drying of the Hb film and showed an absorption spectrum (559 and 530 nm; the characteristic spectrum of hemochrome) distinct from those before dehydration (Hb: 556 nm, HbO_2: 576 and 542 nm). In our dehydration system, the heme pocket and its peripheral environment were preserved optically. The mechanism of stabilization of dehydrated Hb by protectors is generally unclear, although there are some speculations.[26—31] The mechanical rigidity of a dry network of associated saccharide molecules supports the native conformation of Hb against physical stress.[27,28] The hydroxyl groups of saccharides can replace hydration sites of Hb occupied by hydrogen bonds of water molecules and the hydrogen bonds prevent the conformational changes by preserving the polar group interaction of Hb.[29] Saccharides act as scavengers of deteriorative free radicals which become triggers for the oxidization of Hb.[30,31]

Table 1. Spectrophotometric Properties of Hb Films and Hb Solutions

Hb	Ligand	λ_{max} nm	ε	λ_{max} nm	ε	λ_{max} nm	ε
	—	555	11.4	—	—	430	124.8
Film	O_2	541	12.2	576	12.2	415	112.0
	CO	538	12.2	569	11.8	419	187.1
	—	555	12.5	—	—	430	133.0
Soln	O_2	541	13.5	576	14.6	415	125.0
	CO	538	13.4	569	13.4	419	191.0

Spectrometric results in the Soret band of the Hb films were obtained as a function of O_2 and CO partial pressures (Fig. 1(a), (b)). Nearly perfect isosbestic points of the spectra in the conversion from Hb to HbO_2 (381.5 and 447 nm) and the conversion from Hb to

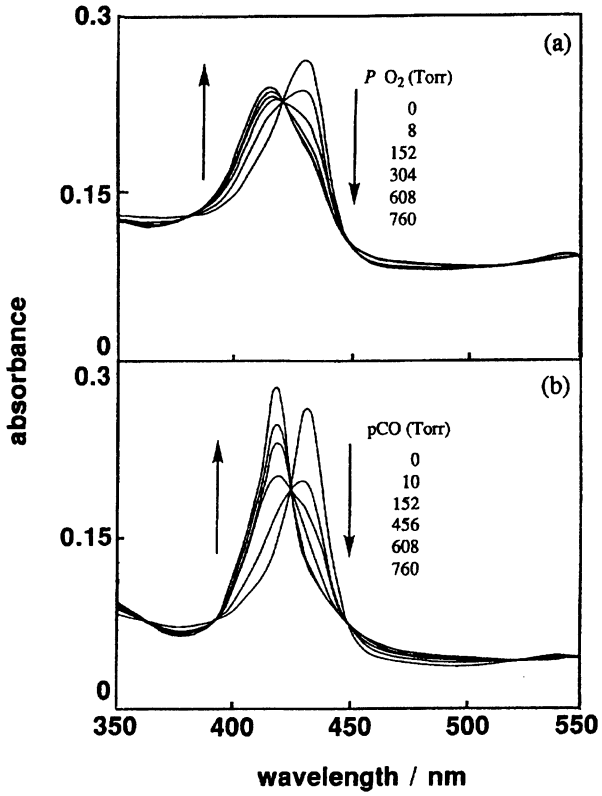

Fig. 1. Change of UV-visible absorption spectrum with increasing (a) O_2 pressure and (b) CO pressure at 22 °C.

HbCO (396 and 451 nm) were observed. This means that the oxygenation and carbonylation of the Hb film occur only as a two-component system, with neither denaturation nor side reactions. Therefore, calibration curves to calculate the oxygenated and carbonylated fractions of Hb could be established linearly by the ratios of the characteristic absorbances of Hb (430 nm), HbO_2 (415 nm), or HbCO (419 nm) from base lines between the isosbestic points.

The binding of O_2 and CO to Hb and the exchange of HbO_2 into HbCO were observed as a function of time (Fig. 2). The O_2 and CO binding reached equilibria at 760 Torr with a 52 and 86% fraction of each ligand gas, respectively. Since both reactions immediately showed 100% saturation in aqueous solutions, we concluded that the ligand reaction is restricted in the solid phase. The initial binding rate of O_2 was faster than that of CO, and the O_2 binding reached equilibrium (11 h) faster than the CO binding (26 h). This tendency is the same as that in aqueous solutions though the rates in the solid phase are lower by several orders of magnitude.

The spectroscopic results of O_2 binding to an Hb film were compared with the result of sorption isotherms of O_2 of the film. The Hb film was transferred quickly from a dry box into an apparatus, deoxygenated at 10^{-6} Torr for 24 h and subjected to 746 Torr of oxygen pressure at 22 °C. The final equilibrium pressure was 742 Torr, the total volume of the container of the apparatus was 132.08 ml, and the amount of O_2 sorbed onto the film at 746 Torr was 2.48×10^{-5} mol, which was 76.3% of the heme amount. In order for us to obtain the amount of physical sorption, a metHb film which was cast from an Hb solution was completely oxidized by a sufficient amount of $K_3[Fe(CN)_6]$. The total fractional amount of oxygen sorbed onto the metHb film which had no ability to bind with O_2 was 30.5%. Therefore, the fractional amount of O_2 binding by heme at 746 Torr O_2 pressure was 45.8%. This value well agrees with the result of UV-visible absorption spectroscopy of the oxygenated Hb film, which shows a fractional saturation of O_2 binding of about 52% at 760 Torr O_2 pressure.

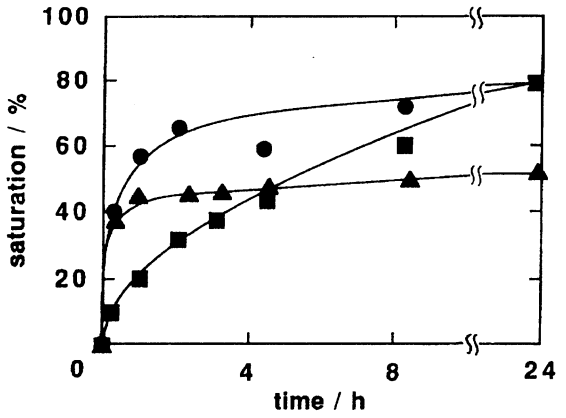

Fig. 2. Ligand binding and exchange of Hb films at 760 Torr and 22 °C. ●, exchange of HbO_2 to HbCO; ▲, O_2 binding; ■, CO binding.

The rate constants (k_{on} and k_{off}) of ligand (O_2 and CO) reactions with Hb films were smaller by about 13 orders of magnitude than those of the corresponding aqueous solutions (Table 2). The binding rate constant (k_{on}) of CO was smaller than that of O_2; however, the releasing rate constant (k_{off}) of CO was substantially smaller than that of O_2, compared to the magnitude of the binding rate. This demonstrates that HbCO is more stable than HbO_2, which agrees with the solution states. The ligand affinities (P_{50}) of Hb films were obtained from the half-saturation points of the binding reactions as a function of the partial pressure from 0 to 760 Torr (Table 2). The P_{50} of O_2 and CO were 760 and 291 Torr, respectively. The higher affinity of Hb for CO than for O_2 in a solid phase is the same as in a liquid phase, but the value is considerably lower than that in the liquid phase. The O_2 binding behavior of the Hb film containing 2.8% water are compared with that of the Hb films containing 8.7% water in Table 2. The Hb film of the higher water content shows the higher rate constants of O_2 binding and dissociation (k_{on} and k_{off}) and the higher affinity for O_2, which consequently induces the higher O_2 saturation at the same O_2 pressure of 760 Torr.

In the solid phase of Hb the ligand coordinates through two steps; one is the diffusion of the ligand through the matrix, and the other is the coordination of the ligand to the heme, which is coupled with the quaternary structural change. The difference in the behavior of ligand-coordination in the solid phase from that in the liquid phase may be induced from one of the two steps or from both. At first, to consider the possibility of a diffusional effect, the ligand reaction of a polymer membrane containing porphyrinatocobalt, which has a ligand reaction without being accompanied by change of the surroundings like the quaternary structural change of proteins, was examined. The binding rate of the complex is high and is similar to that in the solution state;[32] $k_{on}=1.8\times10^{7}$ ($M^{-1}\,s^{-1}$), $k_{off}=3.6\times10^{3}$ (M^{-1}). Therefore, the rate-determining step of the ligand reaction of the membrane is the diffusion step of the ligand through the membrane to the complex. However, the rate of the ligand reaction of the Hb film is too slow to explain based on the ligand diffusion.

Furthermore, only 6.5×10^{-2} s is needed for O_2 to pass through a cellulose nitrate polymer film (this is a model of a maltose membrane) 1.4 μm in thickness, where the diffusion coefficient is supposed to 1.5×10^{-7} $cm^2\,s^{-1}$.[33] Even though the diffusion time is prolonged by incorporation of Hb in the membrane, the time scale of the diffusion rate in the polymer membrane should be faster than that of the ligand reaction of the Hb film. Therefore, the rate-determining step of the ligand reaction in the Hb film is not the ligand diffusion step

Table 2. CO and O_2 Binding and Releasing of Hb Films and Hb Solutions

Hb	Ligand[a]	Water	k_{on}	k_{off}	Saturation	P_{50}
		wt%	$M^{-1}s^{-1}$	s^{-1}	%	Torr
Film	O_2	2.8	7.40×10^{-8}	8.34×10^{-11}	36.7	760
	O_2	8.7	7.42×10^{-7}	0.98×10^{-10}	97.9	—
	CO	2.8	1.34×10^{-8}	4.17×10^{-14}	76.3	291
Soln[b]	O_2	—[c]	2.9×10^{6}	1.8×10^{2}	100.0	40
(T state)	CO	—[c]	2.2×10^{5}	0.9×10^{-1}	100.0	0.3

a) 760 Torr, b) From Ref. 34, c) [Hb]=10 μM.

but the ligand-binding step; that is, the restricted ligand binding will induce a time lag. It may be not the heme itself but the globin which was changed by dehydration to affect the ligand binding of heme, because there was no change in the heme spectra.

It is well known that the ligand binding of heme is coupled with two quaternary structures (the T and R states) of globin and that the ligand binding to a subunit in the T state causes the quaternary structural change to the R state.[34] The quaternary structure of Hb (the T state) is more stable and has a lower affinity for ligand binding than that of HbO_2 (the R state). We suggest that the dynamic conversion of the quaternary structure of the globin which significantly influences the binding-release of ligands is restrained in the solid state.

It is very interesting that the ligand exchange from HbO_2 to HbCO is easier than CO binding to the Hb film (Fig. 2). Because ligand exchange is carried out through dissociation of oxygen from HbO_2 and binding of CO to the resulting Hb, the rate of binding should always be higher than the exchange rate of O_2 with CO. This phenomenon can be explained by the quaternary structural change. HbO_2 and HbCO are R states, while Hb is the T state. In the liquid phase, the ligand exchange is accompanied by two steps of the quaternary structural change, namely from R to T and from T to R, and these changes are very fast. In the solid phase, if there is no structural change during the ligand exchange from HbO_2 to HbCO, namely the R state only, so the rate of ligand exchange should be faster than that of CO binding to Hb because the structural change from T to R is considered to be the rate-determining step.

This hypothesis was confirmed by the absorption spectra of the aromatic region of Hb during the ligand exchange from HbO_2 to HbCO in an Hb film (Fig. 3). The interface of the subunits is affected by the quaternary structural change, that is, β37Trp and α42Tyr of the interface exhibits an absorption peak at 274 nm in the aromatic region of the ultraviolet spectrum. The transition of T to the R state exposes the $\alpha_1\beta_2$ interface, exhibiting an increase in the absorption peaks.[23,35] This absorption spectral change of the globin is independent of the ligand binding of heme and provides quantitative data on the quaternary structure of Hb, namely the R and T states, even in the solid

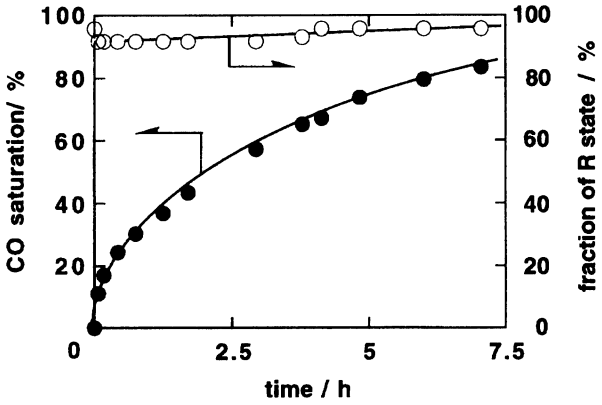

Fig. 3. Ligand exchange of HbO_2 to HbCO and quaternary structural change of Hb in films at 760 Torr of CO, 22 °C. ●, fraction of HbCO; ○, fraction of R state.

phase. The results in Fig. 3 show no change in the quaternary structure as a fixed R state during the progress of ligand exchange. Presumably the restrained quaternary structural change in the solid phase should be the rate-determining step in the solid phase and suppresses the ligand binding.

As shown in Fig. 4, the ligand binding and the quaternary structural change are observed together as a function of time by UV-visible absorption spectra. A film showed oxygenation saturation of 37%, and the transition of the T to the R sate was restricted to about 50%. In this experiment, the Hb film contained 2.8% of water, which was lower than that of the Hb film used in the experiment of Fig. 2. This explains the lower O_2 saturation degree of Fig. 4 in comparison with Fig. 2 (52%). Interestingly, when highly humidified O_2 flows onto this Hb film, the limited O_2 saturation of 37% increases immediately to nearly 100%. The quaternary structure observed in the ultraviolet spectrum also changes immediately to an R state. So both reactions are considerably restrained in the dry state. The quaternary structure which is in the solution state is fixed in the rigid matrix and loses its flexibility. Therefore, the T state restrained in the solid phase will suppress the ligand binding, because of the low O_2 affinity of the T state. However, the addition of a few water molecules restores flexibility to the restrained structure, and the

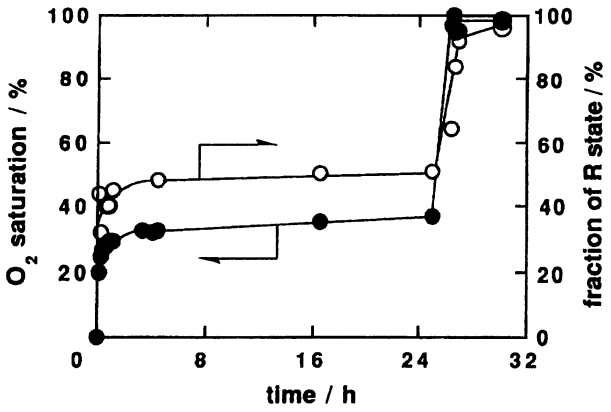

Fig. 4. O_2 saturation and quaternary structural change of the Hb moistured by flowing O_2 humidified in 89% relative humidity (RH) after 25 h. (760 Torr of O_2, 22 °C) ●, fraction of HbO_2; ○, fraction of R state.

potential ability of ligand binding should be activated immediately.

The amount of water which activates the dynamics of Hb structure in the solid phase was studied through the relationship of the oxygen saturation degree and the relative humidity which regulates the water content in the Hb film (Fig. 5). The O_2 saturation degree of the film was increased from 37 to 98% proportionally to the relative humidity from 30 to 89%. The water contents of the films with O_2 saturation levels of 37 and 98% were 2.8 and 8.7 wt%, respectively. The difference in the water content which caused a significant difference in O_2 saturation was very small.

It is well known that the ligand affinities of Hb are modulated by binding and releasing of several small molecules and ions such as H^+, CO_2, phosphates, Cl^-, etc. In the solid phase the binding and the releasing of them should be impossible because of the deficiency of water molecules which hydrate and transfer such small molecules and ions. However, the number of water molecules per Hb molecule observed in Fig. 5 was smaller than the number of water molecules forming the monolayer which covered the surface of an Hb molecule (29 wt%), but was similar to the amount of water molecules hydrating the polar groups of the surface of an Hb (R state) molecule (7.5 wt%).[12] This amount of water is too small for the hydration and the migration of such small molecules and ions. Additionally, there was no dependence of salt concentration in the ligand binding behavior of the Hb film. Therefore, the effect of the binding and releasing of the small molecules and ions is considered to be weak in solid phases. The water molecules to the solid phase would hydrate the polar groups of the surface of an Hb molecule to cleave the hydrogen bonding of maltose, providing the flexibility of the globin. It was reported that in a solution system, the two structures (R and T) had different degrees of hydration; 60 extra water molecules (1.7 wt% relative to Hb) were bound in the transition from Hb in the T state to HbO_2 in the R state.[4] These water molecules bound to the interface of $\alpha_1\beta_2$ became exposed to the aqueous phase on rotation of the subunits, and acted thermodynamically to provide an essential energetic contribution to the transition of Hb into the R state.[4] The hydration degree of the Hb film with 8.7 wt% water content is enough to hydrate the extra surface which is exposed at the R state.

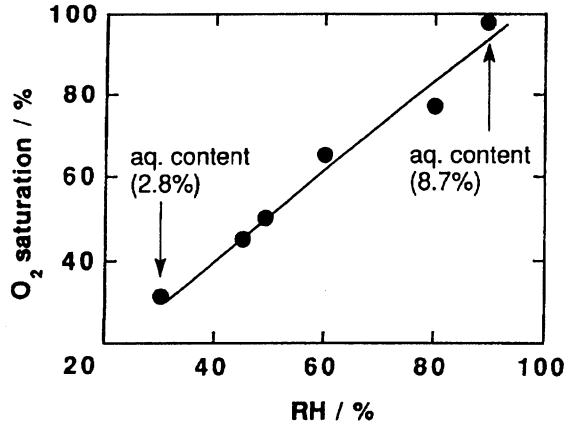

Fig. 5. Relationship between relative humidity (RH) and O_2 saturation of Hb films.

In conclusion, the ligand reaction of Hb films is significantly restricted due to the restrained quaternary structural change of Hb accompanied by the ligand reaction. The dynamic fluctuation of the quaternary structure restrained by dehydration becomes free with the addition of a small amount of water in the film, and the ligand reaction is promoted immediately. The interaction of water with Hb is essential to the activation of the function of Hb, although its structure and conformation are preserved as well as the morphological stabilization. This phenomenon provides some important information on the function of water and the dynamics of proteins in the solid phase.

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