FLASH PHOTOLYSIS STUDIES ON NITRIC OXIDE-FERRIHEMOPROTEIN COMPLEXES

Evidence for the photodissociation into the ferric state

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1. Introduction

In order to study the kinetic behavior of ligand binding, flash photolysis is widely used with various hemoproteins [1–4]. The applicability of this method, however, seems to be limited to the ferrous ligated complexes, such as CO, O_2 and NO complexes, since it has been believed that only those ferrous complexes are photodissociable.

To extend this technique to the ferric state, we have examined the photodissociability of the various complexes of ferrihemoproteins.

2. Materials and methods

Horseradish peroxidase (HRP) was purified from the crude material of Toyobo (Japan) by DEAE- and CM-cellulose column chromatography as in [5]. The enzyme used (RZ = 3.1) was the main fraction from the CM-cellulose column chromatography. The NO complexes of ferrihemoproteins were prepared as follows: nitrogen gas was passed over the surface of the solution of ferrihemoproteins in a cuvette (1 cm light path) for 20 min with occasional stirring. The cuvette was evacuated and then NO gas was introduced to 1 atm. Optical absorption spectra were measured with a Cary-118 spectrophotometer. The concentration of HRP was determined spectrophotometrically by the use of an extinction coefficient at 403 nm of 107 mM^{-1} cm^{-1}. The sample solution was photolyzed by the flash photolysis apparatus consisting of a xenon flash lamp, with a pulse width of 400 μs and an energy of 8 J, equipped with the optical detection system. Fluctuations of the energy with each flash were less than 5%. Temperature was thermostatically controlled at 20 ± 0.1°C.

3. Results

The optical absorption spectra of ferri- and ferro-HRP complexes with NO are shown in fig.1. The NO–ferri-HRP has the typical hemochromogen type spectrum with A_{max} at 565, 538 and 422 nm. Figure 2 shows the time course of the absorption change after photolysis of NO–ferri-HRP, measured at different wavelengths. By photolysis, the A_{405}, a characteristic to ferri-HRP, increases and then decreases slowly with a half-time of 1.7 ms. A similar time-course of ΔA is also seen at 425 nm, near the A_{max} of NO–ferri-HRP.

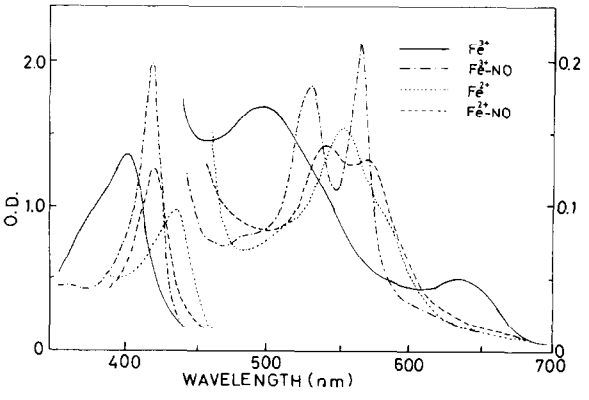

Fig.1. Optical absorption spectra of ferri- and ferro-HRP (Fe^{3+} and Fe^{2+}) and their complexes with NO. Ferro-HRP was obtained by the addition of small amounts of $Na_2S_2O_4$. HRP, 14 μM; phosphate buffer, 0.2 M, pH 7.0.

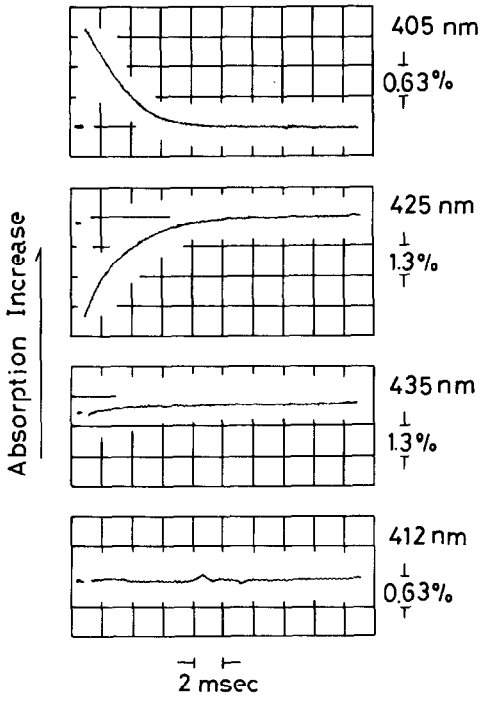

Fig.2. The oscilloscope traces of ΔA by photolysis of NO–ferri-HRP. Phosphate buffer, 0.2 M, pH 7.4. HRP, 10 μM.

The ferro-HRP, however, does not appear photolyzed, as shown by the absence of the ΔA_{435}. No ΔA_{412} is observed, where there is the isosbestic point between ferri-HRP and its NO complex (cf. fig.1). The difference spectrum obtained at 1 ms after flash is shown in fig.3A. The spectrum has an A_{422} min and a broad peak around A_{400} in the Soret region together with distinct A_{538} min and A_{568} min, which is similar to the difference spectrum of NO–ferri-HRP minus ferri-HRP (solid line, fig.3B). Therefore it is concluded that the absorption change shown in fig.2 is due to the photodissociation of the NO complex into the ferric state, followed by the recombination process of NO to ferri-HRP.

The recombination reaction of NO with ferri-HRP obeys the pseudo first-order kinetics because of the high concentration of NO, as shown in the inset of fig.4. From the figure, the second-order rate constant is estimated to be 2×10^5 M^{-1} s^{-1}, which is pH-independent, in spite of the formation of the 'alkaline form' of this enzyme above pH 11.

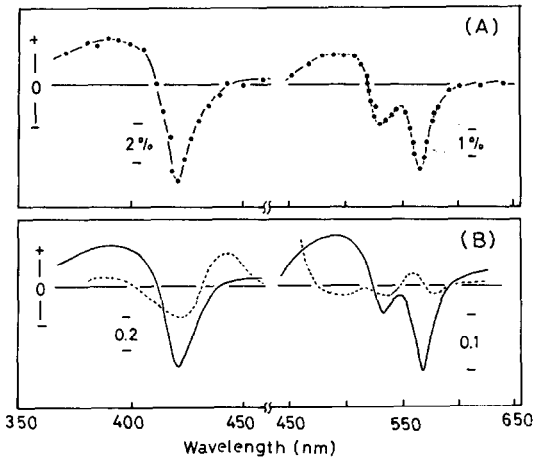

Fig.3. (A) Kinetic difference spectrum of flash photolysis of NO–ferri-HRP. The spectrum is taken at 1 ms after flash. Absorption increases in the upward direction. HRP concentrations used are 10 μM and 30 μM for the Soret and visible regions, respectively. Phosphate buffer, 0.2 M, pH 7.4. (B) Difference spectra of NO–ferri-HRP minus ferri-HRP (solid line) and NO–ferro-HRP minus ferro-HRP (dashed line). Ordinate is absorbance. Phosphate buffer, 0.2 M, pH 7.4. HRP, 14 μM.

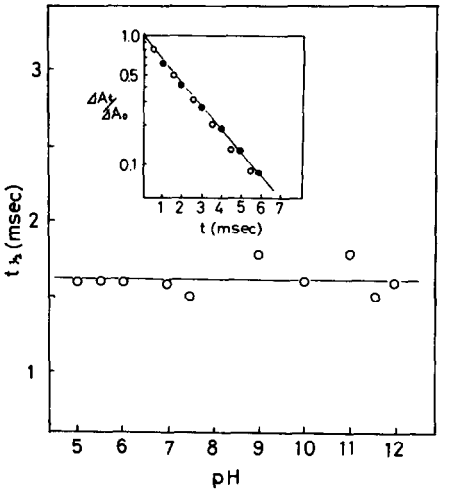

Fig.4. The pH dependence of the half-time or recombination of NO to ferri-HRP. Inset: first-order plots of ΔA_{425} (open circles) and ΔA_{405} (filled circles) calculated from the traces of fig.2. ΔA_t and ΔA_0 are absorption change at any time and total absorption change, respectively. Buffers used are: 0.2 M acetate (pH 5–6); 0.2 M phosphate (pH 6–8); 0.2 M Tris–HCl (pH 8–10); 0.2 M glycine–NaOH (above pH 10).

4. Discussion

The present experimental results demonstrate that the NO complex of ferri-HRP is photodissociable. The product of the photodissociation is the ferric state, not the ferrous state, as verified by the difference spectrum of fig.3. A similar photodissociation into the ferric state was also observed in other NO–ferrihemoproteins; the apparent photodissociability is in the order of

HRP >> lactoperoxidase ≈ metmyoglobin

> methemoglobin.

Here, we have chosen the relatively low energy of flash lamp, because by this energy the ferrous–NO complexes of these hemoproteins were not photolyzed to an appreciable extent. This excluded the possibility of the photodissociation of ferrous–NO complexes formed in the sample, since NO reduces slowly the ferric state to the ferrous state. So far, in our experimental conditions, the photodissociation of other ligands such as CN^-, N_3^- and F^- could not be observed.

It is noted that the optical absorption spectrum of NO–ferri-HRP is a typical hemochromogen type, suggesting Fe^{2+}–NO^+ rather than Fe^{3+}–$NO^\cdot$ for this complex. Taken this into consideration, a possible interpretation for the photodissociation is that the electronic structure of NO-ferri-HRP is similar to that of the photodissociable ferrous complex, such as a CO complex. In any case, the photodissociation of NO–ferrihemoproteins into ferric state may give the critical argument concerning the quantum mechanical interpretation of this phenomenon [6].

The ability of the photodissociation of NO complexes into the ferric state can provide a unique approach to the kinetic studies of ligand-binding. Figure 4 shows the typical example of such studies, where the recombination velocity of NO to ferri-HRP is not affected by pH. This seems puzzling because, above pH 11, the 'alkaline form' of this enzyme appears, which may cause a decrease of the recombination velocity of NO, as observed in the formation of compound I [7]. This result suggests that, after photolysis, recombination of NO into the enzyme is faster than the formation of the 'alkaline form', which is known to be a rather slow process (~ 10 ms half-time) [8]. Furthermore, at pH 12, the difference spectrum obtained at 1 ms after flash is similar to that of fig.3, not to that of the 'alkaline form'. Thus, speculation arises that the conformation of this enzyme appeared after the sudden departure of NO by photolysis differs from the 'alkaline form'. The details will be reported elsewhere.

The presence of the photodissociability in the NO complexes with ferrihemoproteins now opens the possibility of extending the various optical techniques to the ferric state as well as the ferrous state [9,10]. The laser-flash photolysis at room temperature and electron paramagnetic resonance measurements at 4.2°K are being planned with various NO–ferrihemoproteins in our laboratory.

References

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