Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 298-304

COPPER COMPLEXES AT N- AND C-SITE OF OVOTRANSFERRIN: QUANTITATIVE DETERMINATION AND VISIBLE ABSORPTION SPECTRUM OF EACH COMPLEX

Takaki Yamamura, Shizuo Hagiwara, Katsuyoshi Nakazato and Kazuo Satake

Department of Chemistry, Faculty of Science, Science University of Tokyo, 1-3, Kagurazaka, Shinjuku-ku, Tokyo 162, Japan

Received January 9, 1984

SUMMARY: (1) Copper complexes at the two sites of ovotransferrin (Tf) differed markedly in the rate of Cu release by EDTA. (2) During the reaction, λ max of the remaining Cu-Tf complex shifted to red side, while the difference spectrum of $Fe_nCu_{2-n}Tf$ vs. Fe_nTf in which the N-site had been preferentially occupied with Fe had λ max at blue side from that of Cu_2Tf, 440 nm. (3) From these results, the intrinsic spectrum for Cu-complex at each site was assigned: λ max 450 nm for N- and 430 nm for C-site. (4) The differences in the release rate and the spectrum can be used for the identification of the two domains of Tf and for the analysis of metal-binding behavior of each site.

Transferrin, a group of homologous iron-binding proteins, is composed of a single polypeptide chain which is folded up into two compact domains. Each domain has one site capable of binding various metal ions besides iron (1). The two sites do not always behave equivalently (2,3,4), although the amino acid sequences of both the domains are considerably homologous, including the metal binding ligands (5,6). As has been known for long, Fe^{3+}-, Cu^{2+}- and Mn^{3+}-transferrin complexes show the characteristic absorption spectra in the visible region, and their colors are used as conventional indicators for studying metal-binding properties of transferrin (1,7,8). There remains, however, a fundamental problem, whether the absorption spectra for the two sites are identical or not (9-12). In the present study, we developed a convenient method for determining quantitatively the distribution of copper at the two sites and, using this method, revealed a

0006-291X/84 $1.50

significant spectral difference between the two copper complexes at the N- and C-site of ovotransferrin.

MATERIALS AND METHODS

Ovotransferrin (Tf) was prepared from chicken egg white by precipitating viscous components at pH 5.4, then CM-Sepharose (CL-6B, Pharmacia) chromatography of the supernatant using the method of Rhodes et al.(13), and if necessary, the Tf fraction was further subjected to DEAE-Sepharose (CL-6B, Pharmacia) chromatography (a linear gradient of NaCl concentration from 0 to 0.1 M in 0.02 M Tris-HCl buffer at pH 8.0) to remove contaminated proteinase inhibitor (ovoinhibitor (14)). The Tf fraction was treated with acidic citrate to obtain apo-form Tf (15). The N-domain was prepared from the inhibitor free Tf, according to the method of Williams (11). The half-molecule was obtained by gel-filtration of the hydrolysate on Sephadex G-100 and then the preparation was freed of iron by the above treatment and used without further purification. All chemicals were of reagent grade and glassware-distilled water was used. As medium, 0.02 M Tris-HCl buffer at pH 8.0 containing 5 mM $NaHCO_3$ was used for preparing Tf solutions.

Partially iron-saturated ovotransferrin (Fe_nTfs) where the N-site had been preferentially occupied with iron (16) were made by adding definite amounts of 0.02 M $Fe(NH_4)_2(SO_4)_2$ in water to 2.0×10^{-4} M apo-Tf. $Fe_nCu_{2-n}Tf$ ($n < 2$) was prepared from Fe_nTf by adding $CuSO_4$ in water at a ratio of [Cu]/[vacant site: 2-n] = 2.0 so as to saturate the vacant sites (preliminary experiments showed no displacement of Fe with Cu under the conditions used). The protein concentration and n value were determined spectrophotometrically by using absorption coefficients $E^{1\%}_{1cm}$: 0.62 at 465 nm for Fe_2Tf, 0.57 at 440 nm for Cu_2Tf (17), and a molecular weight of 76,600 for Tf (18).

The transfer of Cu from its Tf complex to EDTA was performed at 30 °C by mixing equal volumes of 2.0×10^{-4} M Tf and 0.2 M EDTA in 0.02 M Tris-HCl buffer containing 5 mM $NaHCO_3$ (finally adjusted to pH 8.0 with small amount of conc. NaOH), and the reaction was followed by measuring the absorbance of the remaining Cu-complex of Tf at 440 nm. The spectral change during the reaction was measured with a rapid reaction analyzer (Union model RA-401). Under the conditions used, the spectrophotometry was scarcely influenced by Cu-EDTA produced.

RESULTS AND DISCUSSION

When Cu_2Tf was incubated with a large excess of EDTA as a accepting chelate, the characteristic absorbance of Cu-Tf complex decreased in a biphasic manner composed of two exponentials and at last Cu^{2+} was completely released from the protein. As the curve (a) in Fig.1 shows, the decrement for each phase was just the same, as far as the change was measured at 440 nm of the peak wavelength for Cu_2Tf. These results indicate that Cu^{2+} is

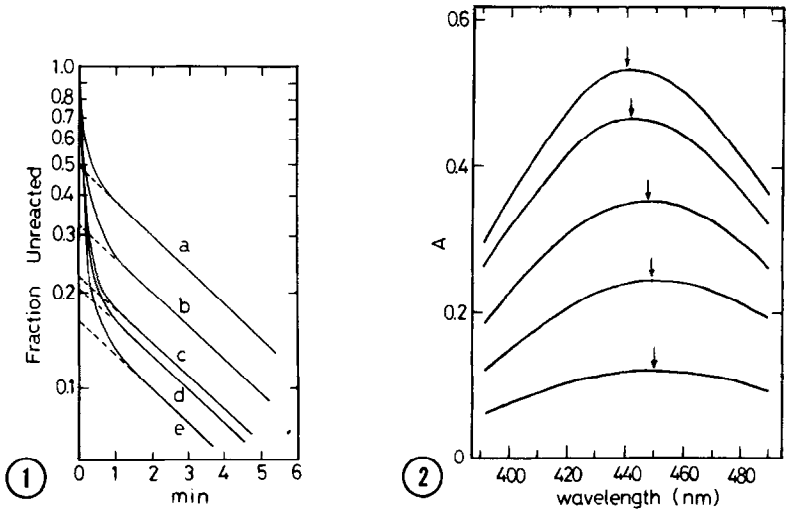

Fig.1(left) Copper-release from $Fe_nCu_{2-n}Tf$ ([Tf] = 1.0×10^{-4} M) with M/10 EDTA at pH 8.0 and 30 °C. The n-values for a, b, c, d, and e were 0, 0.6, 0.98, 1.2 and 1.6, respectively.

Fig.2(right) Spectral change of Cu-complex of Tf after rapid mixing of equal volumes of 2.6×10^{-4} M Cu_2Tf and 0.2 M EDTA. Arrows show the peaks of spectra.

released from the two sites at different rates and Cu^{2+}-complexes at both the sites have the same molar extinction coefficient at 440 nm. During the reaction, however, spectrum of the remaining Cu-Tf shifted to the red side up to 450 nm (Fig.2), suggesting the spectral difference between Cu-complexes at the two sites.

For further investigation of the spectral difference, we prepared $Fe_nCu_{2-n}Tf$ from Fe_nTf ($n < 2$) in which the N-site had been preferentially occupied with Fe^{3+} (16). The difference spectrum of $Fe_nCu_{2-n}Tf$ vs. Fe_nTf, which gives the spectrum attributed to the Cu-complex moiety, was not identical with that of Cu_2Tf vs. apo-Tf. Fig.3 shows blue-shift of the spectrum, depending on n in $Fe_nCu_{2-n}Tf$, i.e. depending on the degree of preferential occupation of Cu at C-site. These results indicate Cu-complex at C-site possesses λ max at a shorter wavelength than 440 nm, whereas, at N-site it is at a longer wavelength.

To determine the quantitative distribution ratio x, Cu at C-site/Cu at N-site, in each $Fe_nCu_{2-n}Tf$, the Cu-release reactions

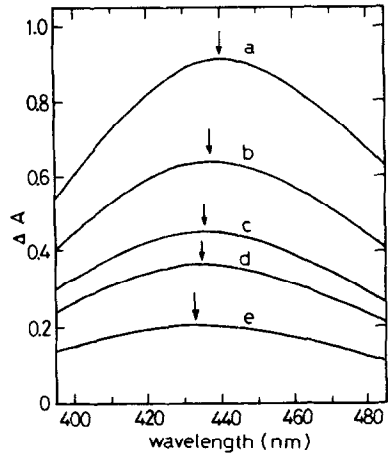

Fig.3 The difference spectrum between $Fe_nCu_{2-n}Tf$ and Fe_nTf. The n-values for a-e are described under Fig.1. Tf concentration was 2.0×10^{-4} M. Arrows show the peaks of spectra.

for these mixed complexes with EDTA were analyzed kinetically. The Fe complex moiety in Tf was stable to EDTA under the conditions used. As summarized in Fig.1 (curves b-e), the reaction proceeded in biphasic manner in this case, too, but the decrement of Cu-complex for slow phase (p) was much less than that for rapid phase (1-p). As the N-site has been preferentially occupied with Fe^{3+} (16), it is clear that the rapid phase corresponds to the Cu-release from C-site and the slow phase to that from N-site. Therefore the x is given by (1-p)/p. Using the x values, 1.0, 2.1, 3.4, 3.7 and 5.1 for n = 0, 0.6, 0.98, 1.2 and 1.6 respectively, and the difference spectra for the mixed complexes (Fig.3), the spectrum intrinsic to the Cu-complex at each site can be assigned by the following analysis. The absorbance of total Cu-complex moiety at a given wavelength λ is represented by

$$A_\lambda = \varepsilon^c_\lambda[\text{Cu-C}] + \varepsilon^n_\lambda[\text{Cu-N}]$$

$$= (\varepsilon^c_\lambda \cdot x + \varepsilon^n_\lambda)\,[\text{Cu-N}] \qquad (1)$$

and on λ = 440 nm, $A_{440} = (\varepsilon^c_{440} \cdot x + \varepsilon^n_{440})\,[\text{Cu-N}]$ (2).

As already mentioned, $\varepsilon^c_{440} = \varepsilon^n_{440} = \frac{1}{2}\varepsilon_{440}$ (3)

where ε_{440} is the molar extinction coefficient of Cu_2Tf at 440 nm. From Eqs. (1)-(3), $$\frac{A_\lambda(1 + x)}{A_{440}} = \frac{2\varepsilon^c_\lambda}{\varepsilon_{440}} \cdot x + \frac{2\varepsilon^n_\lambda}{\varepsilon_{440}} \quad (4)$$ The plot of the left side value of Eq.4 vs. x showed a good straight line with a correlation coefficient > 0.998 for each wavelength λ. The slope and intercept of the straight line give the relative molar extinction coefficient at each λ for Cu-complexes at C- and N-site, respectively. The assigned spectra in the visible region are shown in Fig.4, together with their summation which agreed with spectrum observed on Cu_2Tf. The spectra of Cu-complexes at N- and C-site are very similar in shape, but λ maxima are 20 nm apart: 450 nm for N- and 430 nm for C-site.

The N-domain fragment prepared by us gave the Cu-complex having λ max at 446 nm which was less than the assigned value (450 nm). The Cu-release reaction with EDTA, however, indicated the preparation was contaminated with C-domain to a value of 20%.

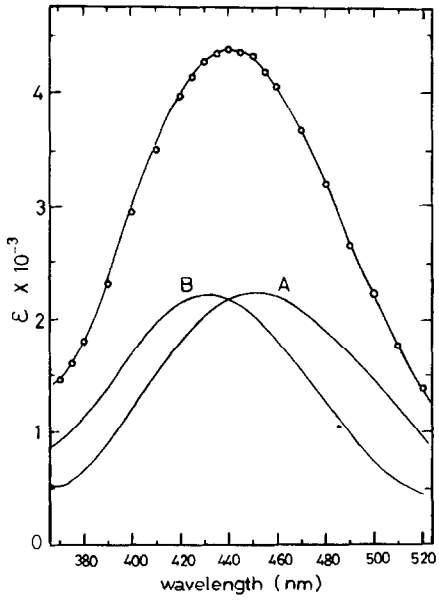

Fig.4 Assigned spectra for Cu-complexes at N- and C-site. A: N-site, B: C-site, o : summation of A and B ; — : observed spectrum of Cu_2Tf. ε at each λ was calculated by usig $E^{1\%}_{1cm}$: 0.57 at 440 nm for Cu_2Tf (17) and M.W. of Tf:76,600 (18).

The simulated spectrum of the Cu-complex composed of N- and C-site at the ratio of 4 : 1 was very similar to the observed one. Williams who succeeded in isolating the N- and C-domain of Tf, reported λ maxima of their Cu-complexes to be 450 nm (11) and 435 nm (12), respectively. The present study shows that this spectral difference between the Cu-complexes is not due to the proteolytic fragmentation of the molecule, but to the intrinsic nature of the two sites.

On Fe- or Mn-complex of Tf, spectral difference in the visible region between the two sites was only slight. Similar slight differences have been also reported by other workers who measured the absorption spectra of iron-loaded fragments of bovine serum transferrin (9) and human serum transferrin (10). The degree of the spectral differences may be due to the hardness, as denoted by Lewis acid, of metal ion concerned. As is well known, Cu^{2+} is much softer than Fe^{3+} or Mn^{3+}. A marked difference between the Cu-complexes at N- and C-site of Tf will be brought about by higher sensitivity of the Cu^{2+}-complex to the microenvironments around their binding sites.

The spectral difference in Cu-Tf affords a convenient criterion not only for the identification of fragmented domains but also for the estimation of the metal-binding behavior of both sites on intact Tf. For example, the stepwise addition of $Al_2(SO_4)_3$ to Cu_2Tf brought about an intense blue-shift of the remaining Cu-complex spectrum, while the stepwise addition of Cu_2SO_4 to apo-Tf showed a slight shift of λ max from red side to 440 nm. These experiments clearly revealed that both the Cu^{2+} and Al^{3+} have higher affinity for the N-site, and Al^{3+} binds more strongly to N-site than Cu^{2+}. The quantitative distribution of Cu at the N- and C-site in such cases can be easily determined from the biphasic Cu-release reaction by EDTA. This method will

be also a useful tool for determining the distribution of other metals (M), including iron, by preparing the mixed complex of $M_nCu_{2-n}Tf$.

REFERENCES

1. Tan, A.T. and Woodworth, R.C. (1964) Biochemistry 8, 3711-3716.
2. Baldwin, D.A. and Deolinda M R de Sousa (1981) Biochem. Biophys. Res. Commun. 99, 1101-1107.
3. Aisen, P., Leibman, A. and Zweier, J. (1978) J. Biol. Chem. 253, 1930-1937.
4. Williams, J., Evans, R.W. and Moreton, K. (1978) Biochem. J. 173, 535-542.
5. Williams, J., Elleman, T.C., Kingston, I.B., Wilkins, A.G. and Kuhn, K.A. (1982) Eur. J. Biochem. 122, 297-303.
6. MacGillivray, R.T.A., Mendez, E., Shewale, J.G., Sinha, S.K., Lineback-Zins, J. and Brew, K. (1983) J. Biol. Chem. 258, 3543-3553.
7. Donovan, J.W. and Ross, K.D. (1975) J. Biol. Chem. 250, 6022-6025.
8. Tomimatsu, Y. and Vickery, L.E. (1972) Biochim. Biophys. Acta 285, 72-83.
9. Brock,J.H. and Arzabe, F.R. (1976) FEBS Lett. 69, 63-66.
10. Zak, O., Leibman, A. and Aisen, P. (1983) Biochim. Biophys. Acta 742, 490-495.
11. Williams, J. (1974) Biochem. J. 141, 745-752.
12. Williams, J. (1975) Biochem. J. 149, 237-244.
13. Rhodes, M.B., Azari, P.R. and Feeney, R.E. (1958) J. Biol. Chem. 230, 399-408.
14. Feinstein, G. and Gertler, A. (1972) Eur. J. Biochem. 31, 25-31.
15. Aisen, P., Leibman, A. and Reich, H.A. (1966) J. Biol. Chem. 241, 1666-1671.
16. Williams, J. and Evans, R.W. (1977) Proteins of Iron Metabolism, pp. 169-177, Grune and Stratton, New York.
17. Fraenkel-Conrat, H. and Feeney, R.E. (1950) Arch. Biochem. 29, 101-113.
18. Warner, R.C. and Weber,I. (1953) J. Am. Chem. Soc. 75, 5094-5101.