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^{15}N NMR STUDIES OF THE BINDING OF ^{15}N-LABELED CYANIDE TO VARIOUS HEMOGLOBINS IN INTACT ERYTHROCYTE

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SUMMARY

^{15}N-labeled cyanide binding to methemoglobins in intact erythrocytes has been studied by ^{15}N NMR. The addition of $C^{15}N^-$ to human and dog hemoglobins in erythrocyte afforded hyperfine-shifted two ^{15}N signals due to the $C^{15}N$ bound to ferric iron of the different heme-units. Single and three distinct signals were observed for rat and rabbit hemoglobins in erythrocyte. These $C^{15}N$ resonance positions are sensitive both to the structural difference in the hemoglobin subunits and to the variety of the animal sources. The $C^{15}N$ spectral difference between solution and intact hemoglobin cyanide is also discussed in relation to a possible change in the intra- and extracellular pH values.

Nuclear magnetic resonance(NMR) spectroscopy has recently developed into powerful means for structural studies of hemoglobin as well as many other hemoproteins in solution(1). Eventually structural studies of the hemoglobin molecule in solution must be related to its function and structure within the intact red blood cell. One of the structural probes for the heme environment in intact hemoglobin molecule has been provided by the ^{13}C NMR of ^{13}C-enriched CO bound to heme-iron in intact erythrocyte(2∿5). However, the two ^{13}CO resonances for the α and β chains of hemoglobin in red cell are sometimes unresolved(3,4) and the ^{13}C chemical shifts are not sensitive to a variety of hemoglobins from different animal sources(4).

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It was also found that the ^{13}CO resonance does not exhibit marked pH dependence in the pH range 6∿8(4,5).

We have currently succeeded in the observation of the ^{15}N resonance of iron-bound $C^{15}N$ in cyanide complexws of various ferric hemin and hemoprotein derivatives in solution(6). The ^{15}N hyperfine shifts of iron-bound $C^{15}N$ have been shown to be quite sensitive to the heme-environmental structures(6). In cyanomethemoglobin, for example, the separation of the $C^{15}N$ resonances bound to the α and β chains was quite large(about 70 ppm)(7), in contrast to the corresponding difference in ^{13}CO resonances(0.4 ppm)(2∿5). Furthermore, the $C^{15}N$ shift has been shown to exhibit pH dependence for various cyanohemoproteins. These situations prompted us to extend our ^{15}N NMR studies for methemoglobin cyanide in intact erythrocytes in order to gain insight into the structural features of intact hemoglobin. We report here some aspects of the $C^{15}N$ shift data of cyanomethemoglobins in blood cells from several animal sources.

MATERIALS AND METHODS

Erythrocytes were prepared from freshly drawn, citrated whole blood(5 ml) from a variety of animal sources. The various animal sources included human, rat(Kyoto; Wister), rabbit (Japanese white) and dog(Beagle). The red cells were collected by centrifugation at 500g, and the packed erythrocytes were washed several times with an isotonic saline solution(0.9% NaCl). The hemoglobins in red cells suspended in the NaCl solution were oxidized with $NaNO_2$ (0.1%) at room temperature for 1 hour(8) and then washed several times with the NaCl solution to remove unreacted $NaNO_2$. Cyanomethemoglobin in blood cell was obtained by the addition of minimum amount of $KC^{15}N$ (Prochem, 96.7% ^{15}N atom)(about 2 mg) and a small portion of red blood cell hemolysate was washed off with minimum volume of isotonic solution. These cyanomethemoglobins in erythrocytes were collected by centrifugation and were transferred into 10 mm NMR tubes(about 2 ml). The suspended solutions of erythrocytes containing slight excess of $C^{15}N^-$ were then at pH 8.4. No detectable hemolysate was found after the ^{15}N NMR measurments (5∿7 hours at 29°C).

The ^{15}N NMR spectra were recorded in a pulse Fourier transform mode with a Jeol PFT-100 spectrometer operating at 10.14 MHz, the deuterium resonance of external D_2O in a 2 mm capillary inserted concentrically into 10 mm tube as a field frequency lock. Typically, 150K transients were collected using 10 KHz spectral width and 4 K data points. The pulse repetition rate of 0.21 sec was employed. Chemical shifts are reported in ppm from external $^{15}NO_3^-$ with the positive sign designated as the downfield shift. The accuracy of the ^{15}N chemical shift is estimated to be ±5 ppm due to the line broadening.

RESULTS AND DISCUSSION

Addition of ^{15}N-labeled KCN to a suspension of erythrocyte containing methemoglobin yielded two broad ^{15}N resonances with large downfield hyperfine shift from the free $C^{15}N^-$ signal (-90 ppm from $^{15}NO_3^-$(9)), located at +970 to +1077 ppm with respect to an external standard $^{15}NO_3^-$. This ^{15}N shift region is characteristic of the ^{15}N resonance of $C^{15}N$ bound to the paramagnetic heme-iron of ferric hemoproteins in solution as shown in our previous work(6,7). Therefore, these large ^{15}N hyperfine shifts apparently indicate that the ^{15}N resoances are not due to the $C^{15}N^-$ ion bound at the cell membrane and/or intracellularly, the presence of which has been evidenced by the ^{13}C NMR study of red blood cells using $^{13}CN^-$(10). In Fig. 1 are demonstrated the ^{15}N NMR spectra of the iron-bound $C^{15}N$ for various cyanomethemoglobins in intact erythrocytes. All the ^{15}N chemical shift data for various intact cyanomethemoglobins are also compiled in Table I, together with those for human adult hemoglobin in solution.

The most interesting feature of the iron-bound $C^{15}N$ resonance for human and dog cyanomethemoglobins in the intact erythrocytes(Fig. 1) is the presence of two distinct $C^{15}N$ signals in the different hemoglobin chains under the intact condition as expected from the corresponding study of human methemoglobin in solution(7). The spacing of these two $C^{15}N$ resonances is large enough (e.g., 41 ppm for dog and 63 ppm

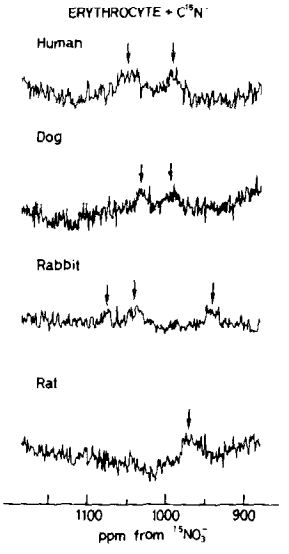

Figure 1: ^{15}N NMR spectra of cyanomethemoglobins in intact erythrocytes using ^{15}N-labeled cyanide. Arrows indicate the iron-bound $C^{15}N$ resonances. From the top, human, dog, rabbit and rat cyanomethemoglobins in erythrocytes, respectively.

for human adult intact hemoglobins) to differentiate the electronic or heme-environmental structures of the α and β heme-units. These are in marked contrast to the observation of unresolved ^{13}CO resonances of human carboxyhemoglobin in blood cells or in whole blood as reported previously(2∿5).

A quite abnormal ^{15}N NMR spectrum was obtained for rat cyanomethemoglobin in intact erythrocyte, where only a single iron-bound $C^{15}N$ resonance was observed. Oxidation of the hemes for rather long periods(about 5 hours) and an addition of an excess of $C^{15}N^-$ to the sample did not yield an additional signal, which may allow us to conclude that the $C^{15}N$ resonances in the two hemoglobin chains are overlapped with each other. This result implies the close similarity of the chemical environments around the heme moieties in the two subunits of rat hemoglobin in erythrocyte.

Table I. ^{15}N chemical shifts of iron-bound $C^{15}N$ for various cyanomethemoglobins in intact erythrocyte and in solution(29°C).

Source	pH	Signal Position[a]	Separation(ppm)
		Erythrocyte	
Human adult	8.4[b]	+1050, +981	63
Dog	8.4[b]	+1031, +990	41
Rabbit	8.4[b]	+1077, +1041, +937	—
Rat	8.4[b]	+970	∿0
		Solution	
	7.3	+1047, +975	72
Human adult	7.7	+1055, +985	70
	8.1	+1083, +1081	65

a) Chemical shifts given in ppm downfield from external $^{15}NO_3^-$, ±5 ppm.
b) Extracellular pH.

Rabbit cyanomethemoglobin is also quite abnormal in that three distinct resonances of the iron-bound $C^{15}N$ were observed in the ^{15}N NMR spectrum. It seems likely that three coordination sites are not provided by the α and β subunits of a single type of hemoglobin molecule. In this regard, Matwiyoff et al. also observed three ^{13}CO resonances bound to hemoglobins from some Duch lop rabbit(3). Accordingly, the hemoglobin heterogeneity concluded from the ^{13}C NMR observation may be responsible for the present ^{15}N NMR anomaly. This hemoglobin heterogeneity appears to be more clearly manifested in the hyperfine-shifted $C^{15}N$ resonances bound to methemoglobin in erythrocyte.

We are now concerned with different features of hemoglobin in intact and solution conditions. This may be revealed by comparing the $C^{15}N$ shift data for cyanomethemoglobin in solution

with those in blood cells. Noteworthy are the $C^{15}N$ resonance positons for human cyanomethemoglobin in erythrocytes under the condition of extracellular pH 8.4, which resemble those of cyanomethemoglobin in solution at pH 7.3(see Table I). At present stage the actual value of the intracellular pH is beyond our knowledge. In this regard Moon and Richards(11) have reported that there is no difference between intra- and extra-erythrocytic pH's in the neutral pH region, which are monitored by the ^{31}P resonance positons of both inorganic phosphate and 2,3-diphosphoglycerate. However, Shulman et al.(12,13) have shown that the intracellular pH is different from the extra-cellular pH in the slightly alkaline or acidic region. For example, when the extracellular pH is 8, the intracellular pH in yeast cell is substantially lower by about one pH unit(13). This appears to be also the case for the human blood cell and the above $C^{15}N$ shift data for hemoglobin in intact erythrocyte are likely indicative of the lower intracellular pH value than the extracellular one. This is, however, based on the assumption that heme-environmental structures of intact and solution hemoglobins are not intrinsically different at the same pH.

Detailed analyses of the differences in the iron-bound $C^{15}N$ shifts between intact and solution hemoglobins in relation to the difference in their structural and/or medium effects should be awaited till much more elaborated ^{15}N NMR studies of cyanomethemoglobin in solution will be done under physiological conditions, for example, in the presence and absence of organic phosphate such as 2,3-diphosphoglycerate. However, we can conclude that the paramagnetic shift of iron-bound $C^{15}N$

resonance for methemoglobin is promissing and pervasive probe to study the molecular properties of hemoglobin under the intact condition.

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