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Characteristics in Tyrosine Coordinations of Four Hemoglobins M Probed by Resonance Raman Spectroscopy†

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ABSTRACT: Resonance Raman spectra of four hemoglobins (Hbs) M with tyrosinate ligand, that is, Hb M Saskatoon (β distal His → Tyr), Hb M Hyde Park (β proximal His → Tyr), Hb M Boston (α distal His → Tyr), and Hb M Iwate (α proximal His → Tyr), were investigated in order to elucidate structural origins for distinctly facile reducibility of the abnormal subunit of Hb M Saskatoon in comparison with other Hbs M. All of the Hbs M exhibited the fingerprint bands for the Fe–tyrosinate proteins around 1600, 1500, and 1270 cm^{-1}. However, Hb M Saskatoon had the lowest Fe–tyrosinate stretching frequency and was the only one to display the Raman spectral pattern of a six-coordinate heme for the abnormal β subunit; the others displayed the patterns of a five-coordinate heme. The absorption intensity of Hb M Saskatoon at 600 nm indicated a transition with a midpoint pH at 5.2, whereas that of Hb M Boston was independent of pH from 7.2 to 4.8. The fingerprint bands for the tyrosinate coordination as well as the Fe–tyrosinate stretching band disappeared for Hb M Saskatoon at pH 5.0, and the resultant Raman spectrum resembled that of metHb A, while those bands were clearly observed for Hb M Boston at pH 5.0 and for two Hbs M at pH 10.0. These observations suggest that the unusual characteristics of the heme in the abnormal β chain of Hb M Saskatoon result from the weak Fe–tyrosinate bond, which allows weak coordination of the proximal histidine, giving rise to the six-coordinate high-spin state at pH 7. At pH 5, the coordination geometry is modified due to protonation of the tyrosinate.

Hemoglobin (Hb)[1] M is a group of mutant Hbs in which the heme iron in the abnormal chain is retained in the ferric state under physiological conditions. The amino acid substitutions of Hbs M are classified into two categories; one includes replacement of the proximal or distal histidine (His) to tyrosine (Tyr), and the other includes replacement of a residue near the distal His of the β chains to glutamate or aspartate (Gerald & Efron, 1961; Heller et al., 1966; Steadman et al., 1970; Cohen-Solal et al., 1973). Hb M Milwaukee (β67Val → Glu) is a representative of the latter category while the former one is further assorted depending on whether the

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[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; RR, resonance Raman; His, histidine; Tyr, tyrosine; ENDOR, electron nuclear double resonance; Val, valine; Glu, glutamic acid.

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mutation occurs in the α or β chain; the α chain mutation at the proximal or distal His gives Hb M Iwate (α87His → Tyr) or Hb M Boston (α58His → Tyr), and the β chain mutation at the proximal or distal His yields Hb M Hyde Park (β92His → Tyr) or Hb M Saskatoon (β63His → Tyr), respectively. Our previous works (Nagai et al., 1980; Nagai & Yoneyama, 1983; Nagai, 1985) have demonstrated that Hb M Saskatoon is distinct from the other three Tyr-coordinated Hbs M; the abnormal chain of Hb M Saskatoon can be reduced by both enzymic and chemical reducing systems at the same rate as normal metHb A, but the other three Hbs M are scarcely reduced by them. In order to elucidate the possible difference in the iron coordination environments of the abnormal subunits between Hb M Saskatoon and the other three Hbs M, we investigated the resonance Raman spectra of four Hbs M.

Resonance Raman (RR) scattering from heme proteins brings about detailed structural information on the heme moiety (Asher, 1981; Rousseau & Ondrias, 1983; Spiro, 1983, 1988; Yu, 1986; Kitagawa & Ozaki, 1987). Some marker bands are sensitive to the coordination number of the heme iron (Spiro et al., 1979; Teraoka & Kitagawa, 1980). On the basis of it, Nagai et al. (1983) previously pointed out, from their RR spectra, that the heme irons in the abnormal subunits of Hb M Iwate and Hb M Boston adopt the five-coordinate structure. In this paper, we examined the RR spectra of Hb M Saskatoon and Hb M Hyde Park under the same experimental conditions as for Hb M Iwate and Hb M Boston and suggest that the heme iron of the abnormal subunit of Hb M Saskatoon is six-coordinated like normal metHb A while those of three other Hbs M are five-coordinated. The pH dependence of RR spectra reveals differences between the Fe–tyrosinate bondings of Hb M Saskatoon and Hb M Boston.

Materials and Methods

Hb M Iwate and Hb M Boston were separated from Hb A by Amberlite CG-50 column chromatography, and Hb M Hyde Park and Hb M Saskatoon were purified from patient's hemolysate by preparative isoelectric focusing (Nagai et al., 1980). The fully oxidized Hbs M were prepared as described previously (Nagai et al., 1980), and their absorption spectra were observed with a Union-Giken SM-401 spectrophotometer. Raman scattering was excited at 488.0 nm with an Ar laser (NEC, GLG3200) and recorded on a JEOL-400D Raman spectrometer equipped with a cooled photomultiplier (RCA 31034a). The temperature of sample solutions was kept at 10 °C during the measurements. The Raman spectrometer was calibrated with indene (Hendra & Loader, 1968).

Results and Discussion

Information on the coordination geometry of the heme iron is obtainable from the ν_3 and ν_{10} modes [mode numbers are based on Abe et al. (1978)]; the ν_3 and ν_{10} modes appear around 1480–1483 and 1610–1620 cm^{-1} for the six-coordinate ferric high-spin state, respectively, and at 1490–1493 and 1628–1630 cm^{-1} for the five-coordinate ferric high-spin state (Spiro & Burke, 1976; Kitagawa et al., 1976; Spiro et al., 1979; Teraoka & Kitagawa, 1980; Ozaki et al., 1986). Figure 1 shows the RR spectra in the 1250–1650-cm^{-1} region of Hb M Saskatoon (A), Hb M Hyde Park (B), Hb M Boston (C), Hb M Iwate (D), and Hb A (E). The RR spectral patterns of Hb M Saskatoon and Hb A have some similarities to each other but are different from those of Hb M Hyde Park, Hb M Boston, and Hb M Iwate, which have similarities to each other. The ν_{10} and ν_3 bands of Hb M Saskatoon were observed at 1607 and 1479 cm^{-1}, respectively, similar to those of Hb A (1611 and 1478 cm^{-1}), indicating that the heme iron adopts

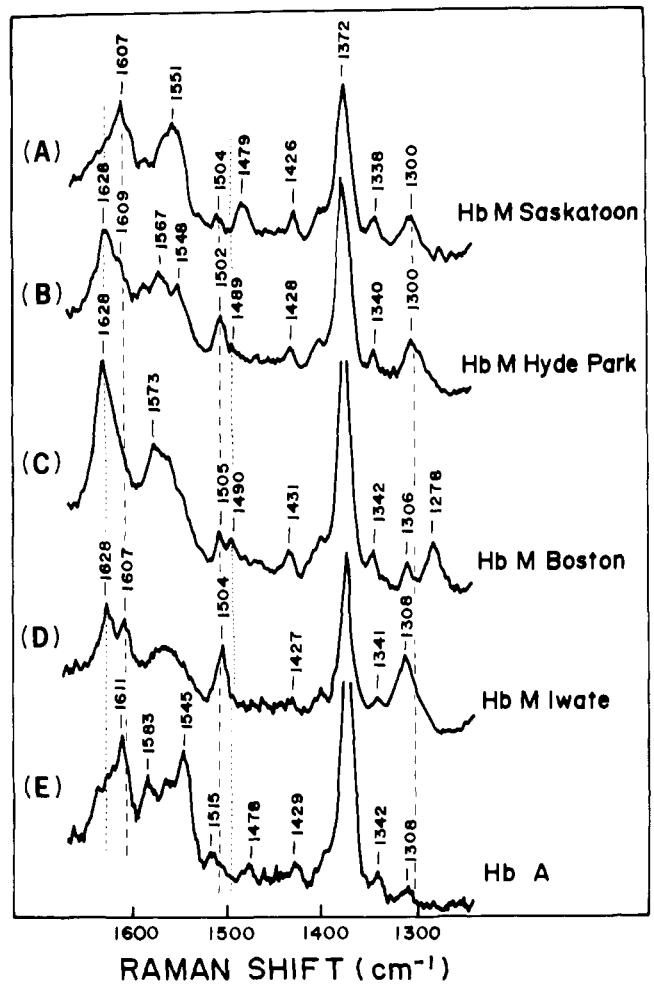

FIGURE 1: RR spectra in the 1250–1650-cm^{-1} region of Hb M Saskatoon (A), Hb M Hyde Park (B), Hb M Boston (C), Hb M Iwate (D), and Hb A (E) in the oxidized form. The broken lines indicate the band positions of the internal vibrations of the coordinated tyrosinate, and the dotted lines represent the ν_{10} and ν_3 band positions expected for the five-coordinate ferric high-spin hemes. All Hbs are in 0.05 M Bis-Tris buffer at pH 7.0 containing 0.1 M NaCl. Heme concentrations are 300 μM except that for Hb M Boston, for which it was 200 μM. Excitation, 488.0 nm.

the six-coordinate structure. On the other hand, the ν_{10} and ν_3 bands of Hb M Hyde Park were observed at 1628 and 1489 cm^{-1}, respectively, similar to those of Hb M Boston (1628 and 1490 cm^{-1}), indicating that the heme irons of the abnormal subunits of Hb M Hyde Park and Hb M Boston adopt the five-coordinate structure.

The X-ray crystallographic study on Hb M Boston (Pulsinelli et al., 1973) demonstrated that the heme iron of the abnormal α subunit is bound to the substituted Tyr but not to the proximal His. For Hb M Iwate, the low-resolution X-ray analysis (Greer, 1971) suggested the coordination of both distal His and substituted Tyr to the heme iron in the abnormal α subunit. However, optical absorption spectra of the abnormal subunits of Hb M Boston and Hb M Iwate are alike (Nagai et al., 1983), and furthermore, the electron nuclear double resonance (ENDOR) study on Hb M Hyde Park and Hb M Iwate (Kankakee) did not display the coupling between an unpaired electron of the heme iron and the ^{14}N nucleus of proximal His, which should be observed as for metHb A and Hb M Milwaukee (Feher et al., 1973), if proximal His were coordinated to the heme iron. All these facts are consistent with the present conclusion that, among the four Tyr-coordinated Hbs M, only Hb M Saskatoon assumes the six-coordinate high-spin structure of heme.

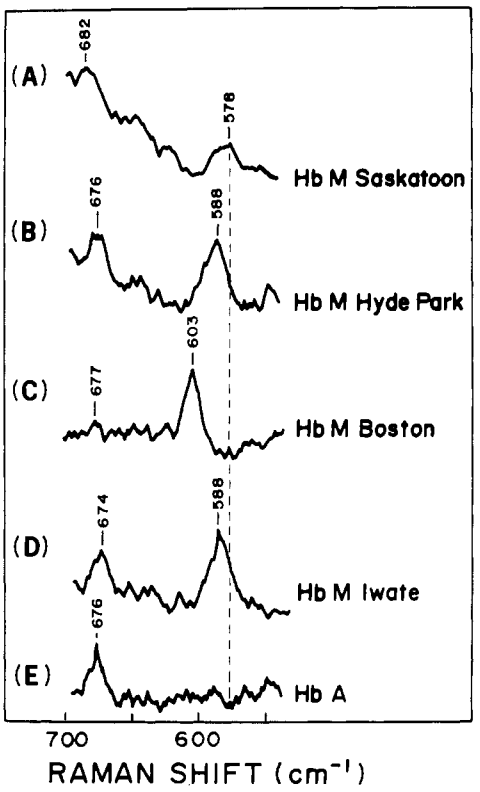

FIGURE 2: RR spectra in the 530–700-cm^{-1} region of Hb M Saskatoon (A), Hb M Hyde Park (B), Hb M Boston (C), Hb M Iwate (D), and Hb A (E). Experimental conditions are the same as those for Figure 1.

Previously Nagai et al. (1983) pointed out that the internal vibrations of the coordinated Tyr yield extra RR bands at 1603, 1504, and 1279 cm^{-1} for Hb M Boston and at 1606, 1507, and 1308 cm^{-1} for Hb M Iwate besides the usual RR bands of an iron porphyrin. These are confirmed in the present study, and the corresponding bands are observed at 1609, 1502, and 1300 cm^{-1} for Hb M Hyde Park and at 1607, 1504, and 1300 cm^{-1} for Hb M Saskatoon, although the 1607-cm^{-1} band of the latter is overlapped with the ν_{10} band. These bands have been regarded as the characteristic fingerprint for tyrosinate coordination to metal ions (Que, 1988). The Tyr band around 1270–1310 cm^{-1} is considered to arise mainly from the C–O stretching mode (Tomimatsu et al., 1976), and its frequency is lowest with Hb M Boston and highest with Hb M Iwate among the four Hb M species examined.

The RR spectra in the lower frequency region of the same five species as for Figure 1 are shown in Figure 2. The ν_7 mode of metalloporphyrins is observed around 674–682 cm^{-1} for all five species. However, the band around 580–600 cm^{-1} is missing with Hb A and accordingly is considered to be related to tyrosine coordination. On the basis of $^{54}Fe/^{56}Fe$, $^{18}O/^{16}O$, and H/D isotope substitution experiments for Fe–cresolate compounds (Pyrz et al., 1985), this mode was assigned to the Fe–O stretching mode coupled appreciably with the phenolate ring vibrations. For Hb M Boston, since this frequency is particularly high and the C–O stretching frequency is particularly low, the σ donation from the tyrosinate oxygen to the ferric iron is considered to be largest among the four Hbs M. In contrast, the frequency of this mode of Hb M Saskatoon is the lowest, suggesting that its Fe–O interaction is relatively weaker than those in Hb M Iwate and Hb M Hyde Park.

When the Fe–tyrosinate interaction is stronger, the heme iron is probably more displaced toward the tyrosinate, and accordingly the sixth ligand, if present, would be more destabilized by increased repulsion with the pyrrolic nitrogens due to the closer contact. Consequently, it seems reasonable that Hb M Saskatoon with the relatively weak Fe–tyrosinate interaction among the four Hb M species contains the six-coordinate heme in the abnormal subunit. It was unexpected that the Fe–tyrosinate bond strengths of Hb M Iwate and Hb M Hyde Park are alike, because the oxygen binding properties in their normal subunits exhibit considerable differences due to interactions with the abnormal subunits (Nishikura et al., 1975). Small differences in the frequencies of the marker bands for the Tyr coordination between the two Hbs M may imply some differences in coordination geometry of the phenolate ring, which result in slightly different tertiary structures and thus variant strengths of salt bridges at the intersubunit surfaces.

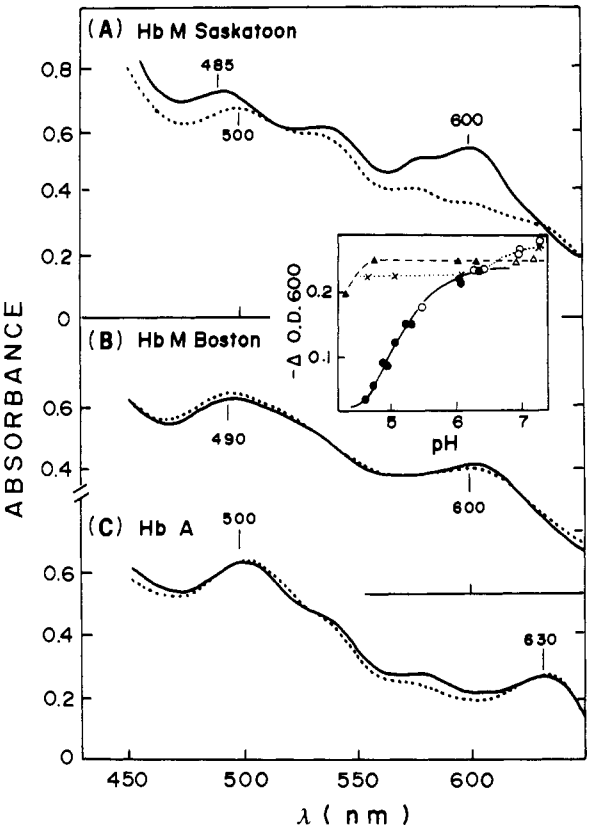

FIGURE 3: Visible absorption spectra of metHb M Saskatoon (A), metHb M Boston (B), and metHb A (C) at pH 7.0 (—) and pH 5.0 (···). All Hbs are in 0.05 M Bis-Tris buffer containing 0.1 M NaCl for pH 7.0 and 0.1 M citrate phosphate buffer for pH 5.0. Heme concentrations were 55 μM for Hb M Boston and 70 μM for Hb M Saskatoon and Hb A. The inset figure shows the pH titration curves for the absorbance at 600 nm for metHb M Saskatoon (○), metHb M Boston (△), and metHb A (×). Buffers used are 0.1 M citrate phosphate (closed symbols) and 0.1 M phosphate (open symbols). Heme concentrations are 70 μM.

In order to get insight into structural features of the heme proximity of Hb M Saskatoon, effects of pH on absorption and RR spectra were investigated in comparison with those of Hb A and Hb M Boston. The absorption spectra in the 450–650-nm regions of Hb M Saskatoon (A), Hb M Boston (B), and Hb A (C) at pH 5.0 and 7.0 are depicted in Figure 3. All of them displayed a large change at pH 10.0 (not shown), but this is mainly due to the change from the aquomet to hydroxymet form of the normal chains, which we do not discuss in this paper. Hb M Saskatoon displayed clear spectral changes at pH 5 while Hb M Boston and Hb A did little changes. For Hb M Saskatoon the absorption maximum at 485 nm was red-shifted to 500 nm, and absorbance at 600 nm,

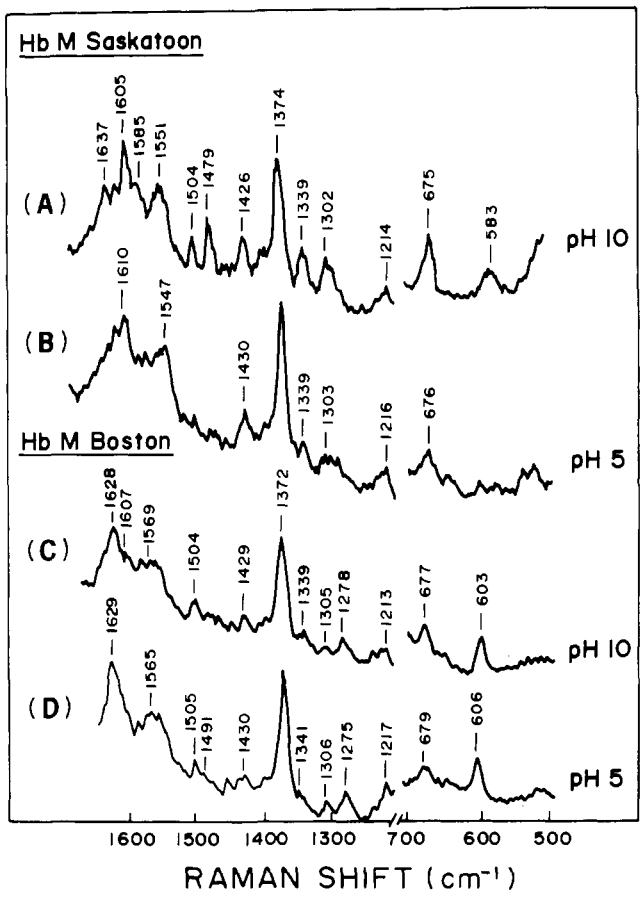

FIGURE 4: RR spectra of Hb M Saskatoon (A and B) and Hb M Boston (C and D) at pH 10.0 (A and C) and 5.0 (B and D) excited at 488.0 nm. Buffers used are 0.1 M glycine–NaOH for pH 10.0 and 0.1M citrate phosphate for pH 5.0. Heme concentrations are 200 μM.

which appears to be characteristic of tyrosinate coordination, greatly decreased at pH 5.0, and the resultant spectrum resembled that of Hb A. These spectral changes were completely reversible, although Hb M Saskatoon and Hb A were denatured at pH 4.3.

The absorbance at 600 nm of Hb M Saskatoon is plotted against pH in an inset of Figure 3. The spectral change occurs in two steps. The transition with the higher pK_a is also seen for Hb A as depicted by a dotted line and therefore is attributed to the normal chain. The other transition takes place with a midpoint pH at 5.2 for Hb M Saskatoon. Hb M Boston exhibits no change until pH 4.8, but after this pH the absorbance gradually decreased until pH 4.3, followed by an abrupt change at pH 4.0.

Figure 4 shows the RR spectra in the 500–700- and 1200–1700-cm^{-1} regions of Hb M Saskatoon and Hb M Boston at pH 10.0 and 5.0. The RR spectrum of Hb M Saskatoon at pH 10.0 gives extra bands at 1637 and 1585 cm^{-1} in comparison with that at pH 7.0 (Figure 1) and a shift of the Fe–tyrosinate stretching mode to a higher frequency (583 cm^{-1}). The bands at 1637 and 1585 cm^{-1} were also observed for Hb A at pH 10.0 (not shown) and ascribed to the formation of the six-coordinate ferric low-spin species (hydroxymet form) in the normal subunits (Iizuka & Yonetani, 1972; Ozaki et al., 1976). The RR bands due to the abnormal chains at 1605, 1504, 1479, and 1302 cm^{-1} are sharpened without a frequency shift. This suggests that the six-coordinate high-spin structure with the tyrosinate as an axial ligand is retained and becomes more firm and thus more homogeneous at pH 10.0. In contrast, at pH 5.0, the marker bands at 1504 and 1479 cm^{-1} disappeared, and the bands at 1303 and 583 cm^{-1} were greatly weakened in intensity. The band at 1605 cm^{-1} is shifted to 1610 cm^{-1}, and the overall RR spectrum of Hb M Saskatoon at pH 5.0 closely resembles that of metHb A at pH 7.0 shown in Figure 1E. These observations strongly suggest that the coordinated tyrosinate is dissociated at pH 5.0.

On the other hand, the RR spectra of Hb M Boston at pH 10.0 and 5.0 are alike. The Fe–tyrosinate stretching frequency is unaltered until pH 5.0. The spectral change of the normal subunit occurs at pH 10.0, but it is not obvious because for Hb M Boston the contribution of the abnormal subunit to the intensity of the RR spectrum is much greater than that of the normal subunit. Anyway, it became evident that tyrosinate is still coordinated to the heme iron for Hb M Boston at pH 5.0 while it is dissociated for Hb M Saskatoon, and therefore, the titration curve shown in the inset of Figure 3 is considered to reflect dissociation of the coordinated tyrosinate.

At pH 7.0 the heme iron in the abnormal chain of Hb M Saskatoon is interacting mainly with the substituted tyrosinate and weakly with the proximal His, but upon a lowering of the pH to 5, the situation is reversed; the coordinated tyrosinate is protonated and its interaction with the heme iron is greatly reduced, but the coordination of the proximal His to the heme iron becomes stronger. Accordingly, the heme iron adopts another six-coordinate structure with the tyrosine at trans to His. Usually a tyrosine residue in proteins has its pK_a in a range from 9 to 12 (Fersht, 1985), but the corresponding pK_a values of the substituted tyrosine in Hb M Saskatoon and Hb M Boston are unusually low. This is due to stabilization of the deprotonated form by coordination to the ferric iron and implies that the distal sides of the heme in the two abnormal chains have strongly ionic environments. X-ray analysis of oxyHb A (Shaanan, 1982) revealed that the distal His of the α subunit can form a stronger hydrogen bond to the bound oxygen than that of the β subunit because of closer contact with the bound oxygen. On the analogy of the distal His, the tyrosyl phenolate of Hb M Saskatoon substituted with the distal His of the β chain might be located further from the heme iron than that of Hb M Boston with the α substitution, causing weaker Fe–tyrosinate bonding and thus allowing the coordination of another ligand at its trans position. We presume that this difference in the iron coordination environments is related to the observed difference in RR spectra, pK_a values, and susceptibilities for the reduction of abnormal subunits between Hb M Saskatoon and Hb M Boston.

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Registry No. Tyr, 60-18-4; Hb M Saskatoon, 9035-07-8; Hb M Hyde Park, 9088-23-7; Hb M Boston, 39340-61-9; Hb M Iwate, 9035-03-4; Fe, 7439-89-6.

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Solid-Phase Synthesis and High-Resolution NMR Studies of Two Synthetic Double-Helical RNA Dodecamers: r(CGCGAAUUCGCG) and r(CGCGUAUACGCG)†

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ABSTRACT: Ten-micromole solid-phase RNA synthesis has been successfully performed on an automated nucleic acid synthesizer with coupling efficiencies up to 99%, using the *tert*-butyldimethylsilyl group to protect the 2′-hydroxyl. The *tert*-butyldimethylsilyl group was easily removed by tetrabutylammonium fluoride under conditions in which virtually no 2′- to 3′-isomerization was found to occur. By use of this approach, the self-complementary RNA dodecamers r(CGCGAAUUCGCG) and r(CGCGUAUACGCG) were synthesized on an automated nucleic acid synthesizer, purified by TLC, and studied by high-resolution NMR. Imino protons were assigned from one-dimensional nuclear Overhauser effects. The nonexchangeable base, H1′, and H2′ protons were assigned by the sequential NOESY connectivity method. The NOE data from these two oligomers were analyzed qualitatively and compared to the ideal A- and B-type helix models of Arnott et al. (1972a,b). The internucleotide H6/H8 NOEs to the preceding H1′ in r(CGCGUAUACGCG) were found to be sequence-dependent and probably reflect the roll angles between adjacent bases. The internucleotide H6/H8 to H2′ NOEs of these oligomers correspond very well to an A-type conformation, but the interstrand adenine H2 NOEs to the following H1′ were much stronger than those predicted from the fiber model. These srong interstrand NOEs can be rationalized by base pair slide to favor more interstrand base overlap, as predicted by Callidine and Drew (1984).

The number of structural studies on synthetic DNA fragments has increased markedly in the last few years, by both X-ray crystal analysis (Dickerson et al., 1982; Wang et al., 1981; Shakked et al., 1983; Nelson et al., 1987) and 2D NMR methods (Hare et al., 1983; Scheek et al., 1983; Feigon et al., 1983; Wemmer et al., 1984a,b; Nilsson et al., 1986; Nilges et al., 1987; Kintanar et al., 1987). Single-crystal X-ray diffraction studies have revealed A-type, B-type, and left-handed Z-type gross DNA conformations as well as local structure variations induced by particular base sequences (Dickerson, 1983). At a lower level of detail, X-ray fiber

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