Synthesis and Characterization of the Dinucleoside Monophosphates Containing 2′-Fluoro-2′-deoxyadenosine†

Seiichi Uesugi, Yoko Takatsuka, Morio Ikehara,* Doris M. Cheng, Lou S. Kan,* and Paul O. P. Ts'o

ABSTRACT: Three dinucleoside monophosphates containing 2′-fluoro-2′-deoxyadenosine (dAfl), dAfl-dAfl, dAfl-A, and A-dAfl, were synthesized chemically. Characterization of these dimers has been performed by UV absorption, CD, and ^{1}H NMR spectroscopies. The results showed that all three dimers have a stacked conformation with a geometry similar to that of A-A but with a greater extent of base–base overlapping than A-A. This interpretation is verified by the data of chemical shifts and coupling constants of ^{1}H NMR study on these dimers (D. M. Cheng, L. S. Kan, P. O. P. Ts'o, S. Uesugi, Y. Takatsuka, and M. Ikehara, unpublished data). All three dimers form 2U·1A complexes with poly(uridylic acid). The T_m of dAfl-dAfl·2poly(U) is higher than that of dAfl-A·2poly(U), which in turn is higher than that of A-dAfl·2poly(U). The NMR results clearly indicate that the conformation of the furanose of dAfl moiety in these dimers is more favored toward 3′-endo than 2′-endo puckering in comparison with the adenosine. The effects of 2′ substituents on oligo- and polynucleotide conformation are also discussed.

We have been studying oligonucleotides containing synthetic nucleoside residues for elucidation of the factors governing oligonucleotide conformation. In the past, our interest has been concentrated mainly on the effects of glycosidic torsion angle. Thus the oligomers containing cycloadenosine and cyclouridine residues were examined in order to elucidate the effects of glycosidic torsion angles fixed in anti to high anti regions (Uesugi et al., 1977, and references therein; Dhingra et al., 1978; Ikehara et al., 1980). The dinucleoside monophosphates containing 8-substituted adenosines were examined also to elucidate the effects of glycosidic torsion angle in the syn region (Ikehara et al., 1972, 1978c; Uesugi et al., 1978). However, the effect of the sugar structure and conformation on the oligonucleotide conformation is also another topic to be studied. It is well-known that the difference of sugar puckering (2′-endo for B-form DNA and 3′-endo for RNA; Arnott & Hukins, 1973; Arnott et al., 1969) is one of the major differences in the monomer units of DNA and RNA which produce major differences in polymer conformation as a whole. Recently, a variety of 2′-substituted 2′-deoxypurine nucleosides (Ikehara & Maruyama, 1978; Ikehara et al., 1978d; Ikehara & Miki, 1978) and the homopolymers containing these residues (Ikehara et al., 1976; Fukui et al., 1977; Ikehara et al., 1977a,b, 1978a,b) were synthesized in our laboratory. It was found that the double-stranded complex of poly(cytidylic acid) with poly(2′-azido-2′-deoxyinosinic acid) (Fukui et al., 1977) and with poly(2′-fluoro-2′-deoxyinosinic acid) (Ikehara et al., 1978b) can induce interferon in several cell lines (De Clercq et al., 1978a,b; E. De Clercq, personal communication). This is the first example of interferon induction by a nonribo double-stranded complex. Furthermore, poly(2′-fluoro-2′-deoxyadenosine) was found to be a superior messenger or template in cell-free protein biosynthesis (T. Fukui and M. Ikehara, unpublished data) or in viral reverse transcriptase (E. De Clercq, personal communication) systems than poly(adenylic acid) itself. We found that the sugar puckering conformation of 2′-substituted 2′-deoxyadenosine derivatives (Ikehara et al., 1978d; Ikehara & Miki, 1978) varies greatly with its substituent as studied by ^{1}H and ^{13}C NMR (Uesugi et al., 1979; S. Uesugi and M. Ikehara, unpublished data; D. M. Cheng, L. S. Kan, P. O. P. Ts'o, S. Uesugi, Y. Takatsuka, and M. Ikehara, unpublished data). 2′-Fluoro-2′-deoxyadenosine (**1**, dAfl)[1] has an unusually high population (~70%) of 3′-endo puckering among nucleosides, while adenosine, 2′-deoxyadenosine, and other derivatives have a much lower population of 3′-endo form (<40%). From ^{1}H NMR (Ts'o et al., 1969; Ezra et al., 1977; Lee et al., 1976) and X-ray crystallographic (Arnott et al., 1969; Seeman et al., 1976; Rosenberg et al., 1976) analyses, it is known that a nucleoside residue in well-stacked oligoribonucleotides favors 3′-endo-furanose puckering. For instance, a dinucleoside monophosphate containing 2′-azido-2′-deoxyadenosine, dAn_3-dAn_3, revealed very similar properties with those of A-A, and this may be predicted by the conformation of the furanose ring in dAn_3, which is similar to that of adenosine (Ikehara et al., 1979). Therefore, it is of great interest to investigate the conformational properties of oligonucleotides containing dAfl residues whose furanose ring has a great tendency to adopt a 3′-endo conformation. In this paper, we report the synthesis and properties of three A-A (**10**) analogues containing dAfl and A residues, dAfl-dAfl (**7**), dAfl-A (**8**), and A-dAfl (**9**) (Figure 1), and discuss the effects of 3′-endo sugar puckering on oligonucleotide stacking conformation.

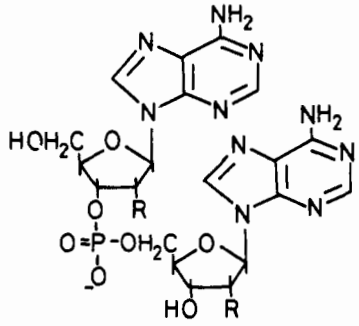
Materials and Methods

General Procedures. UV absorption spectra were recorded on a Hitachi 323 spectrophotometer. CD spectra were recorded on a JASCO ORD/UV-5 spectropolarimeter equipped with a CD attachment and a thermojacketed cell. The instrument was calibrated with an aqueous solution of *d*-10-camphorsulfonic acid. The molar ellipticity, $[\theta]$, is presented in terms of per residue value. The continuous variation experiments and melting experiments were carried out on a Cary

† From the Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565, Japan (S.U., Y.T., and M.I.), and the Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205 (D.M.C., L.S.K., and P.O.P.T.). *Received September 29, 1980.* This research was supported in part by a Japanese Ministry of Education Grant-in-Aid for Scientific Research. This is paper 60 in the series Polynucleotides. For paper 59, see Ohtsuka et al. (1980).

[1] Abbreviations used: dAfl, 2′-fluoro-2′-deoxyadenosine; NMR, nuclear magnetic resonance; DMF, dimethylformamide; DCC, dicyclohexylcarbodiimide; CD, circular dichroism; DSS, sodium 4,4-dimethyl-4-silapentane-1-sulfonate; TLC, thin-layer chromatography; UV, ultraviolet; MeOTr, monomethoxytrityl; Bz, benzoyl; other abbreviations principally follow the recommendation of the IUPAC–IUB Commission of Biochemical Nomenclature (1970 and 1977).

0006-2960/81/0420-3056$01.25/0



7, $R_1 = R_2 = F$ dAfl-dAfl
8, $R_1 = F, R_2 = OH$ dAfl-A
9, $R_1 = OH, R_2 = F$ A-dAfl
10, $R_1 = R_2 = OH$ A-A

FIGURE 1: Structures of the dinucleoside monophosphates.

14 and Varian 219 spectrophotometer, respectively. The molar extinction coefficient of poly(U) is 9.2×10^3 at 265 nm. ^1H NMR spectra were recorded on a Bruker WH-360 (360 MHz) spectrometer operating in the Fourier-transform mode. Chemical shifts are measured in parts per million from DSS. Paper chromatography was performed on Whatman 1 paper with the following solvent systems: solvent A, 2-propanol–concentrated NH_4OH–water (7:1:2); solvent B, ethanol–1 M ammonium acetate (pH 7) (7:3); solvent C, 1-butanol–acetic acid–water (5:2:3); solvent D, 1-propanol–concentrated NH_4OH–water (55:10:35). Paper electrophoresis was performed for 1 h with a voltage gradient of 35 V/cm on Toyo filter paper No. 51A by using 0.05 M triethylammonium bicarbonate buffer (pH 7.5). Thin-layer chromatography was performed on silica gel plates with Merck Kieselgel HF_{254} by using mixed solvent systems of chloroform and ethanol. Snake venom phosphodiesterase was purchased from Worthington Biochemical Corp., and the incubation was carried out in 0.1 M NH_4HCO_3 at 37 °C for 5 h with the enzyme (0.2 mg/mL).

5′-O-Monomethoxytrityl-dAfl (**2**). dAfl (**1**) (269 mg, 1 mmol) was rendered anhydrous by repeated evaporation with anhydrous pyridine. The final residue was dissolved in DMF (5 mL), and monomethoxytrityl chloride (377 mg, 1.2 equiv) was added. The mixture was stirred until a clear solution was obtained. The solution was kept at room temperature for 2 days. After disappearence of the starting material by TLC ($CHCl_3$–ethanol, 8:1), the reaction mixture was poured into ice–water containing 2% concentrated NH_4OH. The precipitates were collected by filtration, washed with water, and dissolved in $CHCl_3$ (30 mL). The $CHCl_3$ solution was washed with water (20 mL) to remove the starting material, dried with anhydrous $MgSO_4$, and evaporated to dryness. After evaporation with anhydrous pyridine, the residue was dissolved in a small volume of ethyl acetate. The product was precipitated with hexane and dried over P_2O_5. The yield was 410 mg (0.76 mmol, 76%); TLC ($CHCl_3$–C_2H_5OH, 8:1) R_f 0.59 (**1**, 0.07). The spot on a plate developed a yellow color on spraying with 30% H_2SO_4 and heating.

N,N,3′-O-Tribenzoyl-dAfl 5′-Monophosphate (**4**). Pyridinium dAfl 5′-monophosphate (Ikehara et al., 1978a) (**3**) (12 660 A_{258} units, 0.89 mmol) was rendered anhydrous by repeated evaporation with anhydrous pyridine. The final residue was suspended in anhydrous pyridine (15 mL), and benzoyl chloride (2.5 mL) was added under cooling in an ice bath. The mixture was shaken well, and the resulting solution was kept at room temperature for 1 h. The reaction mixture was poured into ice–water (50 mL), and the product was extracted with $CHCl_3$. After being washed with water, the $CHCl_3$ fraction was evaporated to dryness. After evaporation with anhydrous pyridine, the residue was treated with acetic anhydride (5 mL) in anhydrous pyridine (15 mL)[2] at room temperature for 24 h. After evaporation of the solvent, 50% aqueous pyridine was added to the residue under cooling in an ice bath. The mixture was kept at room temperature for 2 h and evaporated to dryness at reduced pressure. The residue was rendered anhydrous by repeated evaporation with pyridine and precipitated with ether from its solution in anhydrous pyridine to give 13 320 A_{280} units of **4** (0.61 mmol, 69%): $UV_{max}^{50\%EtOH}$ 234 and 273 nm (sh); PC R_f (B) 0.75; PEP R_m (pA-A) 0.67.

N,N,3′-O-Tribenzoyl-dAfl (**5**). To a solution of **2** in pyridine (10 mL) cooled in an ice bath was added benzoyl chloride (2 mL). The mixture was kept at room temperature for 4 h and poured into aqueous $NaHCO_3$ solution with stirring. The benzoylated compound was extracted with $CHCl_3$. After being washed with aqueous $NaHCO_3$ solution and water, the $CHCl_3$ fraction was evaporated to dryness. The residual pyridine was removed by coevaporation with toluene, and the residue was treated with 80% aqueous acetic acid at room temperature overnight. After evaporation of the solvent, the residual acetic acid was removed by coevaporation with water, and then the residual water was removed by coevaporation with pyridine. The product was precipitated with hexane from its solution in ethyl acetate. The yield was 460 mg (0.79 mmol, 81%): $UV_{max}^{50\%EtOH}$ 230, 255 (sh), and 274 nm (sh); TLC ($CHCl_3$–C_2H_5OH, 20:1) R_f 0.38. This compound gave a negative result in the color test for the trityl group.

dAfl 3′-Monophosphate (**6**). A mixture of pyridinium cyanoethyl phosphate (0.3 mmol) and **2** (108 mg, 0.2 mmol) was rendered anhydrous by repeated evaporation with anhydrous pyridine and dissolved in anhydrous pyridine (3 mL), and DCC (206 mg, 1 mmol) was added. The mixture was kept at room temperature for 2 days. After 50% aqueous pyridine was added, the mixture was kept at room temperature overnight. Dicyclohexylurea was removed by filtration, and the filtrate was extracted with hexane. The aqueous solution was evaporated to dryness at reduced pressure. After removal of the residual pyridine by coevaporation with toluene, the residue was treated with 80% aqueous acetic acid (20 mL) at 50 °C for 1 h. After removal of the solvent and the residual acetic acid by coevaporation with water, the residue was dissolved in 50% aqueous pyridine and extracted with ether. After evaporation of the aqueous fraction, the residue was treated with concentrated NH_4OH at 50 °C for 2 h. After removal of volatile materials, the residue was dissolved in aqueous pyridine, washed with $CHCl_3$, and evaporated to dryness. The residue was dissolved in 0.01 N HCl and applied to a column of charcoal for chromatography (1 × 7 cm).[3] After the column was washed with water, elution was carried out with 50% aqueous C_2H_5OH containing 5% concentrated NH_4OH. After removal of the solvent, the residue was dissolved in water (15 mL) and applied to a column of Dowex 1-X2 ($HCOO^-$ form, 1 × 4 cm). After the column was washed with water and 0.05 N HCOOH, the desired product was eluted with 0.1 N HCOOH. Formic acid was removed by repeated coevaporation with water. The yield was 1500 A_{256} units (0.1 mmol, 50%): $UV_{max}^{H^+}$ 256 nm; PEP R_m (pA-A) 1.06. The ^1H NMR data are consistent with the assigned structure (D. M. Cheng, L. S. Kan, P. O. P. Ts'o, S. Uesugi, Y. Takatsuka, and M. Ikehara, unpublished data).

[2] This treatment is to remove the benzoyl group on the phosphate residue (Lapidot & Khorana, 1963).

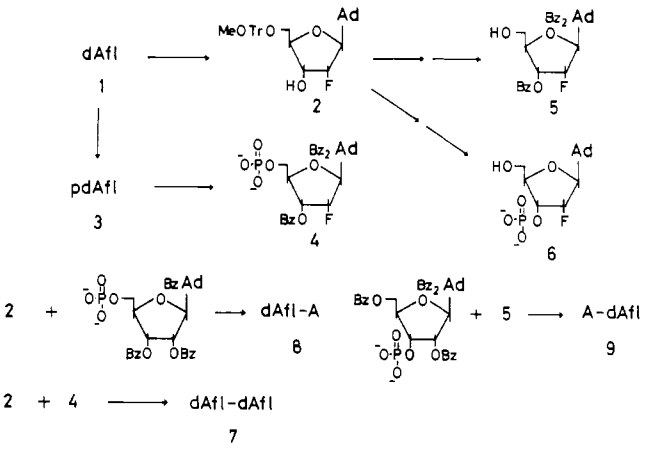
[3] Activated charcoal for chromatography (Code No. 031-02135) supplied by Wako Pure Chemical Industries, Ltd., Osaka, was used.

dAfl-dAfl (**7**). A mixture of **2** (271 mg, 0.5 mmol), the pyridinium salt of **4** (13 320 A_{280} units, 0.61 mmol), and pyridinium Dowex 50 resin[4] was rendered anhydrous by repeated evaporation with pyridine and dissolved in anhydrous pyridine (3 mL), and DCC (1.26 g, 6.1 mmol) was added. The mixture was kept at room temperature for 5 days, and 50% aqueous pyridine was added under cooling in an ice bath. The mixture was kept at room temperature overnight. Dicyclohexylurea and Dowex resin were removed by filtration. After evaporation of the filtrate and removal of the residual pyridine by coevaporation with toluene, the residue was treated with 80% acetic acid (50 mL) at room temperature overnight. The solid material was removed by filtration, and the filtrate was evaporated to dryness. The residual acetic acid was removed by coevaporation with water, and water was removed by evaporation with pyridine. The residue was treated with methanolic ammonia at 30 °C for 1 day. After evaporation of the volatile materials, the residue was dissolved in aqueous pyridine, washed with ether, and evaporated to dryness. The residue was dissolved in water (150 mL) and applied to a column of DE 23 cellulose (HCO_3^- form, 1.8 × 40 cm). After the column was washed with water, elution was carried out with a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) (0–0.15 M, total 4 L). The fractions of the second peak, which contained the desired product and some byproduct, were pooled and desalted by repeated evaporation with water. A solution of the residue in water (50 mL) was applied to a column of Dowex 1-X2 ($HCOO^-$) (1.5 × 19 cm). Elution was carried out with a linear gradient of HCOOH (0–0.15 N, total 3 L). The second peak contained pure dAfl-dAfl (**7**). The yield was 3010 A_{256} units (0.12 mmol, 24%). Formic acid was removed by repeated evaporation with water and lyophilization. The various properties are shown in Tables I and II and Figure 2.

dAfl-A (**8**). A mixture of **2** (287 mg, 0.53 mmol), pyridinium *N*,2′,3′-*O*-tribenzoyladenosine 5′-phosphate (14 060 A_{280} units, 0.64 mmol), and pyridinium Dowex 50 resin (0.5 mL) was rendered anhydrous by repeated evaporation with pyridine and then taken up in anhydrous pyridine (3 mL), and DCC (660 mg, 3.2 mmol) was added. The mixture was kept at room temperature for 6 days. The reaction mixture was worked up in the same manner as the case of dAfl-dAfl. After treatment with methanolic ammonia, the final residue was dissolved in water (150 mL) and applied to a column of DE 23 cellulose (HCO_3^- form, 1.8 × 40 cm). After the column was washed with water, elution was carried out with a linear gradient of triethylammonium bicarbonate buffer (pH 7.5) (0–0.15 M, total 4 L). The fractions of the second and third peaks, which were partially resolved, were pooled and desalted by repeated evaporation with water. The final residue was dissolved in water (30 mL) and applied to a column of Dowex 1-X2 ($HCOO^-$ form, 1 × 3 cm), and elution was carried out with water, 0.05 N HCOOH, and 0.1 N HCOOH successively. The desired product, dAfl-A, was eluted in the 0.1 N HCOOH fraction. The yield was 960 A_{257} units (0.037 mmol, 7%). The poor yield is mainly due to an accidental loss during the workup. The various properties are shown in Tables I and II and Figure 3.

A-dAfl (**9**). A mixture of **5** (311 mg, 0.53 mmol), pyridinium *N*,*N*,2′,5′-*O*-tetrabenzoyladenosine 3′-phosphate (534 mg, 0.63 mmol), and pyridinium Dowex 50 resin (0.5 mL) was rendered anhydrous by repeated evaporation with pyridine and taken up in anhydrous pyridine (3 mL), and DCC (1.3 g, 6.3 mmol) was added. The mixture was kept at room temperature for 5 days and at 30 °C for 3 days. Aqueous pyridine (50%) was added under cooling with ice–water. The reaction mixture was extracted with hexane. A second portion of hexane was added, and the mixture was allowed to stand at room temperature overnight. Dicyclohexylurea and resin were removed by filtration, and the aqueous layer was evaporated to dryness under reduced pressure. The residue was rendered anhydrous by repeated evaporation with pyridine and treated with methanolic ammonia at 30 °C for 1 day. The volatile materials were evaporated off. A solution of the residue in water (150 mL) was applied to a column of DE 23 cellulose (HCO_3^- form, 1.8 × 40 cm). After the column was washed with water and 0.02 M triethylammonium bicarbonate buffer (pH 7.5), elution was carried out with a linear gradient of the same buffer (0.02–0.06 M, total 2 L). The third peak contained the desired product, A-dAfl (1480 A_{258} units, 0.061 mmol, 12%). The various properties are shown in Tables I and II and Figure 4.

Scheme I



Results and Discussion

Synthesis. dAfl-A (**8**) was synthesized by condensation of 5′-*O*-monomethoxytrityl-dAfl (**2**) and benzoylated pA. The synthesis of dAfl-dAfl (**7**) is similar to that of **8** except benzoylated pA is replaced by benzoylated pdAfl (**4**). A-dAfl (**9**) was synthesized by condensation of benzoylated Ap and dAfl protected with benzoyl groups except for 5′-OH (**5**). A brief description of the synthesis and yield of these protected dAfl derivatives is given below. **2** was synthesized by the reaction of dAfl (Ikehara & Miki, 1978) with monomethoxytrityl chloride in DMF with a yield of 76%. **4** was synthesized by benzoylation of pdAfl (**3**; Ikehara et al., 1978b) with benzoyl chloride with a yield of 69%. Benzoylation of **2** with benzoyl chloride in pyridine and detritylation of the product with 80% acetic acid gave **5** in 81% yield. dAfl 3′-phosphate (**6**), which was used as a reference compound for ^1H NMR analysis, was synthesized by phosphorylation of **2** with cyanoethyl phosphate (Tener, 1961) and DCC. Deblocking and isolation by Dowex 1 column chromatography gave **6** in 50% yield. A list of all these reactions is summarized in Scheme I.

7 was synthesized by condensation of **2** and **4** with DCC in pyridine at room temperature for 5 days. After the solution was deblocked, isolation was tried by DEAE-cellulose column chromatography, but a byproduct which showed $\lambda_{max}^{pH7.5}$ at 259 and 305 nm was also found in the peak containing the desired product. Pure **7** was obtained by column chromatography on Dowex 1 under acidic condition with a yield of 24%. The byproduct may be a N^6-phosphorylated compound. **8** was synthesized by condensation of **2** and *N*,2′,3′-*O*-tribenzoyl-pA

[4] The resin was added to ensure removal of a trace of amine possibly present in the reaction mixture.

Table I: Chromatographic Properties of the Dinucleoside Monophosphates

compound	paper electrophoresis,[a] R_m(pA-A)[b]	paper chromatography,[a] R_f in solvent A	B	C	D
dAfl-dAfl	0.53	0.51	0.52	0.32	0.60
dAfl-A	0.52	0.42	0.46	0.21	0.54
A-dAfl	0.46	0.51	0.52	0.25	0.57
pdAfl	1.06	0.19	0.36	0.23	0.44
dAfl	0.00	0.81	0.69	0.61	0.72
pA	1.00	0.14	0.30	0.15	0.37
A	0.00	0.76	0.62	0.46	0.66

[a] Composition of buffer and solvent systems are given under Materials and Methods. [b] Relative mobility to pA (1.0) and adenosine (0.0).

Table II: Ultraviolet Absorption Data of the Dinucleoside Monophosphates

compound	pH	λ_{max} (nm)	ϵ(P) at λ_{max}[a]	hypochromicity[b] (%)
dAfl-dAfl	2	256.5	26 000	
	7	257	22 700	17
	12	257	21 800	
dAfl-A	2	257	26 100	
	7	257	23 800	14
	12	257.5	24 000	
A-dAfl	2	257	26 300	
	7	258	24 100	15
	12	258	24 400	

[a] Determined by phosphorus analysis and given in ϵ per phosphate residue. UV spectra were measured at room temperature (≃20 °C). [b] Calculated from hydrolysis experiment with snake venom phosphodiesterase. UV spectra were measured at room temperature (≃20 °C).

under similar conditions. A byproduct similar to that in the case of **7** was again produced. Successive column chromatography on DEAE-cellulose and Dowex 1 resin gave pure **8**. **9** was synthesized by condensation of *N,N*,2′,5′-*O*-tetrabenzoyl-Ap and **5**. After deblocking, the product was isolated by DEAE-cellulose column chromatography with a yield of 12%. A summary of these reactions is also collected in Scheme I. All these dimers could be completely hydrolyzed by snake venom phosphodiesterase to give corresponding nucleoside and nucleoside 5′-phosphate in a 1:1 ratio. Chromatographic properties (Table I), UV absorption spectra (Figures 2–4 and Table II), phosphorus analysis[5] (Table II), and ^{1}H NMR spectra (Figure 6) all support the dimer structures to be correct.

UV and CD Studies. All the dimers show some hypsochromic shift of λ_{max} by 2–3 nm with respect to the monomer (Table II). Hypochromicity was calculated from the result of hydrolysis with snake venom phosphodiesterase (Table II). dAfl-dAfl shows significantly larger hypochromicity (17%) than A-A (12%) (Kondo et al., 1970). dAfl-A and A-dAfl show hypochromicities (14% and 15%, respectively) which are also larger than that of A-A but smaller than that of dAfl-dAfl. These results suggest that all the dimers containing dAfl are more stacked than A-A, and dAfl-dAfl is the most stacked one. CD spectrum of dAfl-dAfl at pH 7 and 20 °C (Figure 2) shows a pattern similar to that of A-A (Kondo et al., 1970; Van Holde et al., 1965; Warshaw & Cantor, 1970), a positive band at 271 nm, a negative band at 251.5 nm, and a positive band at 218.5 nm. However, the magnitudes of the bands for

[5] Phosphorus analysis was carried out by a combined method of Baginski et al. (1967) and Chen et al. (1956).

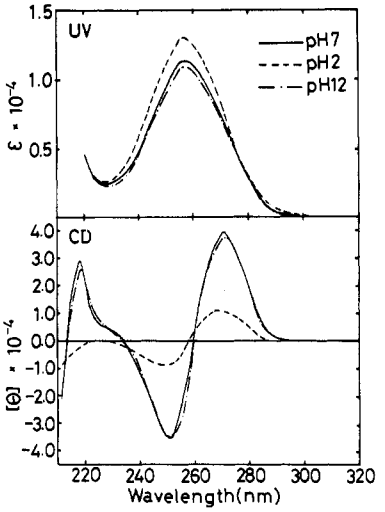


FIGURE 2: UV absorption and CD spectra of dAfl-dAfl in 0.01 N HCl (pH 2), 0.01 M sodium cacodylate buffer (pH 7), and 0.01 N NaOH (pH 12) containing 0.1 M NaCl at 20 °C.

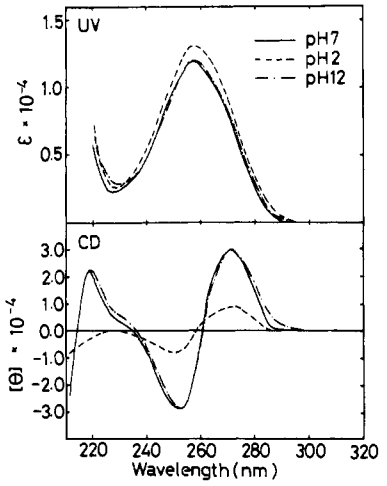


FIGURE 3: UV absorption and CD spectra of dAfl-A in 0.01 N HCl (pH 2), 0.01 M sodium cacodylate buffer (pH 7), and 0.01 N NaOH (pH 12) containing 0.1 M NaCl at 20 °C.

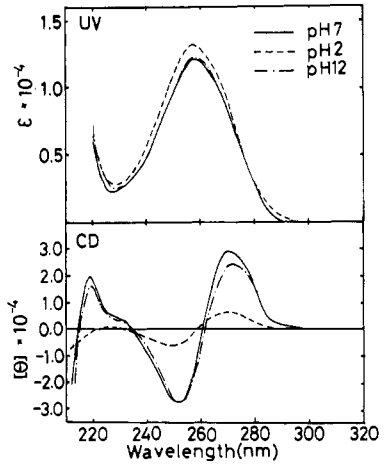


FIGURE 4: UV absorption and CD spectra of A-dAfl in 0.01 N HCl (pH 2), 0.01 M sodium cacodylate buffer (pH 7), and 0.01 N NaOH (pH 12) containing 0.1 M NaCl at 20 °C.

dAfl-dAfl are much larger than those for A-A. The $[\theta]_{max}$ of the positive band at around 270 nm are 3.9×10^4 for the former and $\sim 2 \times 10^4$ for A-A under similar conditions. dAfl-A and A-dAfl show almost identical spectra at pH 7 and 20 °C, and patterns similar to those of dAfl-dAfl and A-A, but the magnitudes of the CD bands in the spectra are between

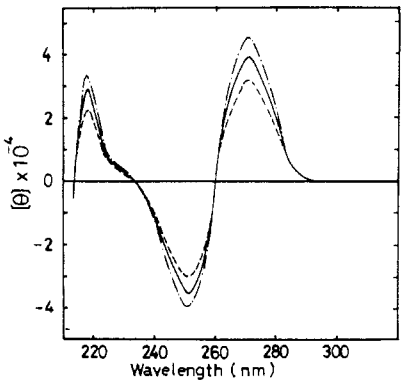


FIGURE 5: CD spectra of dAfl-dAfl at 5 °C (-·-), 20 °C (—), and 35 °C (---) in 0.1 M NaCl and 0.01 M sodium cacodylate buffer (pH 7).

those of dAfl-dAfl and A-A (Figures 3 and 4). The $[\theta]_{max}$ of the positive band at around 270 nm (3.0×10^4 for dAfl-A and 2.9×10^4 for A-dAfl) are about 1.5 times as large as that of A-A. These results are consistent with the notion that the dimers containing dAfl take a conformation with an anti,anti, right-handed stack like A-A, and the degree of stacking in these dimers decreases in the order dAfl-dAfl > dAfl-A ≈ A-dAfl > A-A. At pH 2, the magnitudes of CD bands of the dimers containing dAfl are markedly reduced, and the positive band at around 220 nm disappears completely. These spectra are quite similar to that of half-protonated A-A (Topal & Warshaw, 1976). It is known that protonation at the base residues destabilizes stacking interaction in a dimer. Figure 5 shows the effect of temperature on CD spectrum of dAfl-dAfl. Temperature change from 5 to 35 °C reduces $[\theta]_{271}$ by only 30%. In the case of A-A, $[\theta]_{270}$ decreases by 40–50% in the same temperature range (Kondo et al., 1970; Tazawa et al., 1970; Powell et al., 1972). So, the stacking conformation of dAfl-dAfl is more stable against thermal perturbation than that of A-A.

^1H NMR Studies. ^1H NMR spectra of all dAfl-dAfl, dAfl-A, and A-dAfl are shown in Figure 6. The resonances of $H_{1'}$, $H_{2'}$, and $H_{3'}$ of dAfl residue can be distinguished easily from other signals because they are coupled with a fluorine atom (the J values are 17, 52, and 18 Hz, respectively). The $H_{2'}$ resonance is shifted to downfield, also due to the fluorine substituent. The base and sugar proton resonances of these three dimers are assigned as shown in Figure 6 (D. M. Cheng, L. S. Kan, P. O. P. Ts'o, S. Uesugi, Y. Takatsuka, and M. Ikehara, unpublished data). It is known that the ribofuranose ring conformation in a nucleoside residue can be treated as a simple 2'-endo–3'-endo equilibrium (Evans & Sarma, 1974). The population of 3'-endo puckering can be calculated from $J_{1',2'} + J_{3',4'}$ and $J_{3',4'}$ or $J_{1',2'} + J_{3',4'}$ and $J_{1',2'}$ values (Altona & Sundaralingam, 1973). As readily seen from the spectra (Figure 6), the coupling constants between $H_{1'}$ and $H_{2'}$ are quite small (~1 Hz). $J_{1',2'}$ of the monomers, dAflp and pdAfl, are 2.9 and 2.1 Hz which correspond to about 70% and 80% population of 3'-endo puckering (Altona & Sundaralingam, 1973; Lee et al., 1976). When these residues are incorporated into the dimers, they take almost pure 3'-endo puckering form. On the other hand, $J_{1',2'}$ values of pA and Ap residues in dAfl-A and A-dAfl are about 3.9 and 3.5 Hz, which are smaller than those of the corresponding monomers but still larger than even those of pdAfl and dAflp (Figure 6). These $J_{1',2'}$ values of A residues in the heterodimers are nearly identical with those for the corresponding pA and Ap residues in A-A (4.0 and 3.6 Hz; Lee et al., 1976) and correspond to about 60% population of 3'-endo puckering. The complete analysis of the ^1H NMR data of the dimers containing dAfl will be published elsewhere (D. M. Cheng, L. S. Kan, P. O. P. Ts'o, S. Uesugi, Y. Takatsuka, and M. Ikehara, unpublished data).

Interaction with Poly(uridylic acid). All three dimers containing 2'-fluoro group form complexes with poly(U) at 0.8 °C. The mixing curves for dAfl-dAfl, dAfl-A, and A-dAfl with poly(U) (Figure 7) show that complex formation occurs with a stoichiometry of 2U·1A. As shown in Figure 8, the complexes of poly(U) with dimers with the 2'-fluoro group exhibit a thermal transition with a sufficiently defined melting temperature (T_m). The T_m values of dAfl-dAfl·2poly(U), dAfl-A·2poly(U), and A-dAfl·2poly(U) are 13, 9, and 7 °C, respectively. A significant increase in thermal stability of dAfl-dAfl–poly(U) complex is observed as compared to A-A·2poly(U) (T_m was 7 °C under the same conditions). At present, it is not clear whether the 3'-endo preference or the presence of fluorine atom itself has anything to do with the increased thermal stability of the dAfl-dAfl–poly(U) complex.

In conclusion, the dimers with the 2'-fluoro group have more extensive stacking conformation than the dimer with the 2'-hydroxyl group, A-A. Fluorine substitution at the 2' position of adenine nucleoside favors the 3'-endo conformation.

The NMR results concerning the base–base stacking and the furanose conformation are consistent with the results by X-ray crystallographic studies of well-stacked dinucleosides monophosphates, which also show that both sugar residues

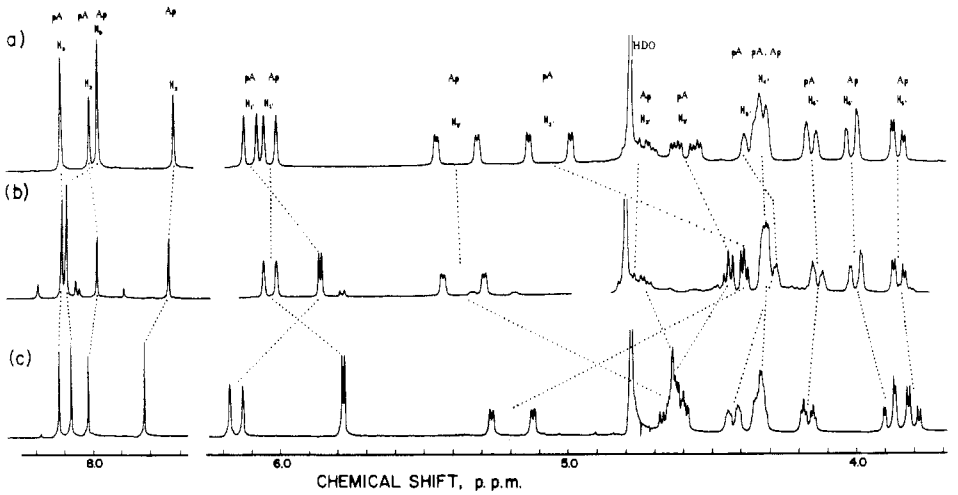


FIGURE 6: 360-MHz ^1H NMR spectra of dAfl-dAfl (a), dAfl-A (b), and A-dAfl (c) in D_2O at pD 7.5 and 25 °C.

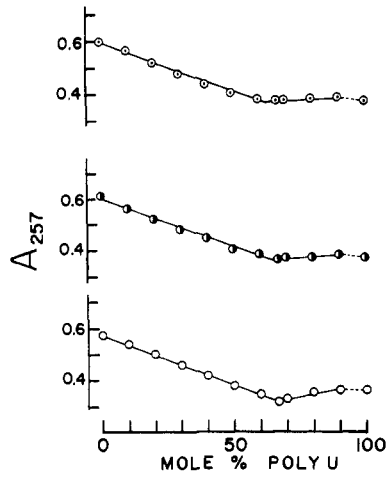


FIGURE 7: Mixing experiment between poly(U) and dAfl-dAfl (○), dAfl-A (◑), and A-dAfl (◎) in 10 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5) at 0.8 °C. The total nucleotide concentration is 5×10^{-5} M.

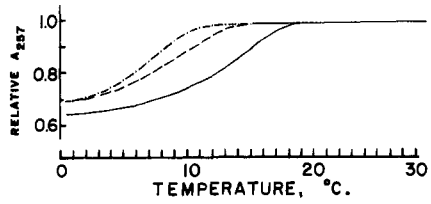


FIGURE 8: Melting curves of poly(U) plus dAfl-dAfl (—), dAfl-A (---), and A-dAfl (-·-) in 10 mM $MgCl_2$ and 10 mM Tris-HCl (pH 7.5). The stoichiometry of each complex is 2U·1A, and the total nucleotide concentration is 5×10^{-5} M.

have 3′-endo puckering. When an A-A molecule is examined with Corey–Pauling–Koltun models, it is immediately obvious that 3′-endo puckering of the 3′-linked nucleoside residue is more favorable for anti,anti, right-handed stacking of the bases since the 2′-OH in 2′-endo puckering provides a steric hindrance against the approach of the 5′-linked nucleoside residue. As to the effect of 3′-endo puckering in the 5′-linked nucleoside residue, careful examination of the molecular model reveals that the 5′-linked nucleoside in the 3′-endo form is more advantageous for stacking than that in the 2′-endo form. In a 3′-endo nucleoside, the base is in a pseudoaxial position on the furanose ring, and, therefore, the base and sugar are in a more folded form. In contrast to this, in a 2′-endo nucleoside, the base is in a pseudoequatorial position on the furanose ring, and, therefore, the base and sugar are in a more extended form.[6] If the relative position between the 3′-linked nucleoside residue and the 5′-linked sugar residue is approximately fixed, the base of the 5′-linked nucleoside in the 3′-endo conformation overlaps with the base of the 3′-linked nucleoside more extensively than that in the 2′-endo conformation.

RNA can take only A-form structure, in which the furanose ring has 3′-endo puckering, whereas DNA takes both A- and B-form structures (Arnott, 1970). Our results revealed that the dimer and the polymer (Ikehara et al., 1978a) bearing the 2′-fluoro group, which is more electronegative than OH, can take more stable A-form structure in spite of the smaller size. The OH group has a much larger electronegativity as well as larger size than H. Therefore, the main reason why RNA does not take B-form structure may be that the 2′-OH group produces both steric and electrostatic repulsions between adjacent residues in B-form structure. In addition, the intrinsic nature of the 2′-OH to prefer 3′-endo puckering to 2′-endo puckering, which is also due to the larger electronegativity, may also help the polyribonucleotide to take A-form structure. The biological activities of poly(dAfl) and poly(dIfl) (E. De Clercq, personal communication; T. Fukui and M. Ikehara, unpublished data) may be a reflection of the stable A-form structure of these polynucleotides.

[6] For example, see Figure 36 of Sundaralingam (1973).

References

Altona, C., & Sundaralingam, M. (1973) *J. Am. Chem. Soc. 95*, 2333–2344.

Arnott, S. (1970) *Prog. Biophys. Mol. Biol. 21*, 267–319.

Arnott, S., & Hukins, D. W. L. (1973) *J. Mol. Biol. 81*, 93–105.

Arnott, S., Dover, S. D., & Wonacott, A. J. (1969) *Acta Crystallogr., Sect. B B25*, 2192–2206.

Baginski, E. S., Foa, P. P., & Zak, B. (1967) *Clin. Chem. (Winston-Salem, N.C.) 13*, 326–332.

Chen, P. S., Jr., Torribara, T. Y., & Warner, H. (1956) *Anal. Chem. 28*, 1756–1758.

De Clercq, E., Fukui, T., Kakiuchi, N., & Ikehara, M. (1978a) *J. Pharm. Dyn. 1*, 62–65.

De Clercq, E., Torrence, P. F., Stollar, B. D., Hobbs, J., Fukui, T., Kakiuchi, N., & Ikehara, M. (1978b) *Eur. J. Biochem. 88*, 341–349.

Dhingra, M. M., Sarma, R. H., Uesugi, S., & Ikehara, M. (1978) *J. Am. Chem. Soc. 100*, 4669–4673.

Evans, F. E., & Sarma, R. H. (1974) *J. Biol. Chem. 249*, 4754–4759.

Ezra, F. S., Lee, C.-H., Kondo, N. S., Danyluk, S. S., & Sarma, R. H. (1977) *Biochemistry 16*, 1977–1987.

Fukui, T., Kakiuchi, N., & Ikehara, M. (1977) *Nucleic Acids Res. 4*, 2629–2639.

Ikehara, M., & Maruyama, T. (1978) *Chem. Pharm. Bull. 26*, 240–244.

Ikehara, M., & Miki, H. (1978) *Chem. Pharm. Bull. 26*, 2449–2453.

Ikehara, M., Uesugi, S., & Kobayashi, M. (1972) *Chem. Pharm. Bull. 20*, 2394–2401.

Ikehara, M., Fukui, T., & Kakiuchi, N. (1976) *Nucleic Acids Res. 3*, 2089–2099.

Ikehara, M., Fukui, T., & Kakiuchi, N. (1977a) *Nucleic Acids Res. 4*, 989–1000.

Ikehara, M., Fukui, T., & Kakiuchi, N. (1977b) *Nucleic Acids Res. 4*, 4249–4260.

Ikehara, M., Fukui, T., & Kakiuchi, N. (1978a) *Nucleic Acids Res. 5*, 1877–1887.

Ikehara, M., Kakiuchi, N., & Fukui, T. (1978b) *Nucleic Acids Res. 5*, 3315–3324.

Ikehara, M., Limn, W., & Uesugi, S. (1978c) *J. Carbohydr., Nucleosides, Nucleotides 5*, 163–185.

Ikehara, M., Maruyama, T., & Miki, H. (1978d) *Tetrahedron 34*, 1133–1138.

Ikehara, M., Takatsuka, Y., & Uesugi, S. (1979) *Chem. Pharm. Bull. 27*, 1830–1835.

Ikehara, M., Uesugi, S., & Shida, T. (1980) *Chem. Pharm. Bull. 28*, 189–197.

Kondo, N. S., Holmes, H. M., Stempel, L. M., & Ts'o, P. O. P. (1970) *Biochemistry 9*, 3479–3498.

Lapidot, Y., & Khorana, H. G. (1963) *J. Am. Chem. Soc. 85*, 3857.

Lee, C.-H., Ezra, F. S., Kondo, N. S., Sarma, R. H., & Danyluk, S. S. (1976) *Biochemistry 15*, 3627–3639.

Ohtsuka, E., Fukumoto, R., & Ikehara, M. (1980) *Chem. Pharm. Bull. 28*, 80–84.

Powell, J. T., Richards, E. G., & Gratzer, W. B. (1972) *Biopolymers 11*, 235–250.

Rosenberg, J. M., Seeman, N. C., Day, B. O., & Rich, A. (1976) *J. Mol. Biol. 104*, 145–167.
Seeman, N. C., Rosenberg, J. M., Suddath, F. L., Kim, J. J. P., & Rich, A. (1976) *J. Mol. Biol. 104*, 109–144.
Sundaralingam, M. (1973) in *Conformation of Biological Molecules and Polymers* (Bergmann, E. D., & Pullman, B., Eds.) pp 417–455, Academic Press, New York.
Tazawa, I., Tazawa, S., Stempel, L. M., & Ts'o, P. O. P. (1970) *Biochemistry 9*, 3499–3514.
Tener, G. M. (1961) *J. Am. Chem. Soc. 83*, 159–168.
Topal, M. D., & Warshaw, M. M. (1976) *Biopolymers 15*, 1755–1773.
Ts'o, P. O. P., Kondo, N. S., Schweizer, M. P., & Hollis, D. P. (1969) *Biochemistry 8*, 997–1029.
Uesugi, S., Yano, J., Yano, E., & Ikehara, M. (1977) *J. Am. Chem. Soc. 99*, 2313–2323.
Uesugi, S., Limn, W., & Ikehara, M. (1978) *Chem. Pharm. Bull. 26*, 3732–3737.
Uesugi, S., Miki, H., Ikehara, M., Iwahashi, H., & Kyogoku, Y. (1979) *Tetrahedron Lett.*, 4073–4076.
Van Holde, K. E., Brahms, J., & Michelson, A. M. (1965) *J. Mol. Biol. 12*, 726–739.
Warshaw, M. M., & Cantor, C. R. (1970) *Biopolymers 9*, 1079–1103.

Renaturation of Bovine Erythrocyte Carbonic Anhydrase B Denatured by Acid, Heat, and Detergent†

Leslie F. McCoy, Jr., and Kin-Ping Wong*

ABSTRACT: The renaturations of bovine carbonic anhydrase B denatured by acid, heat, and detergent have been studied in a continuing effort to elucidate the folding mechanism of this protein. Complete loss of enzymatic activity was observed when the native protein was acidified to pH 2, heated to 70 °C, or treated with 0.025% and 0.1% sodium dodecyl sulfate. These denatured proteins possess conformation states which are different from the native conformation and the random-coiled state of the protein. They have been obtained both from the native protein directly and from the random-coiled protein and shown to be the same regardless of the initial protein conformation. The results suggest that the native conformation is not required for the formation of the unique conformations of the partially denatured proteins. The renaturation of the denatured proteins by direct reversal to native conditions generally yields an enzymatically inactive protein with conformations different from the native one, and sometimes results in aggregation and precipitation. The precipitates formed upon renaturation by reversing the pH to neutral or cooling the protein back to room temperature may be states first attained kinetically upon renaturation. The dissolution of the precipitate is then kinetically too slow to enable complete renaturation. However, these denatured proteins can be completely renatured to the native conformation with total recovery of enzymatic activity if they are first converted to the random-coiled state by guanidinium chloride. The results raise the possibility of "irreversible" denaturation of proteins that are exposed to these denaturants during isolation and purification and suggest a general method for renaturing the otherwise "irreversibly" denatured proteins. As part of this work, the high and low detergent-binding states of carbonic anhydrase have been characterized by UV difference spectroscopy, by near- and far-UV circular dichroism spectra, and by intrinsic viscosity. The thermal denaturation profile of this protein was established by the $\Delta pH/\Delta T$ method, and the melting temperature (T_m) was determined to be 64.3 °C.

The last step in protein synthesis is the folding of the newly synthesized polypeptide chain into a unique conformation. The demonstration of the existence of intermediate conformations in the in vitro folding of a random-coiled protein will enable a detailed study of how the genetic information encoded in the amino acid sequence is expressed in the formation of the unique native conformation [e.g., Baldwin (1975) and Wong & Tanford (1970, 1973)]. Such intermediate conformations show which parts of a refolding protein are formed first and indicate what types of interactions stabilize these intermediates. Moreover, they may provide information on the nucleation sites for the folding of other parts of the protein [e.g., Wetlaufer & Ristow (1973)].

Studies on the reversible denaturation of bovine erythrocyte carbonic anhydrase (Wong & Tanford, 1970, 1973; McCoy & Wong, 1979) have shown that the protein can be denatured to the random-coiled state by 6 M guanidinium chloride (GdmCl).[1] Furthermore, they showed that this unfolding–refolding equilibrium transition consists of separable, intermediate steps. Preliminary kinetic investigations of denaturation and renaturation of carbonic anhydrase (Wong & Tanford, 1970, 1973; Yazgan & Henkens, 1972; Wong et al., 1972a,b; Carlsson et al., 1973; Wong & Hamlin, 1975; Ko et al., 1977; Ikai et al., 1979; McCoy et al., 1980) have established the existence of kinetic intermediate states, including an "incorrectly folded" conformation which appears to exist in the transition region.

The acid denaturation of carbonic anhydrase leads to extensive aggregation (Riddiford, 1964; Coleman, 1965; Edsall et al., 1966; Wong & Hamlin, 1974). Subsequent to acid denaturation, efforts to renature the protein by direct reversal

† From the Department of Biochemistry, University of Kansas Medical Center, Kansas City, Kansas 66103 (K.-P.W.), and the Department of Chemistry, University of South Florida, Tampa, Florida 33620 (L.F.M.). *Received May 16, 1980.* Supported by National Institutes of Health Research Grants HL 18905 and, in part, GM 22962.

* Author to whom correspondence should be addressed. K.-P.W. is a research career development awardee of the National Institute of General Medical Sciences (GM 70628).

[1] Abbreviations used: GdmCl, guanidinium chloride; $NaDodSO_4$, sodium dodecyl sulfate; CD, circular dichroism.

0006-2960/81/0420-3062$01.25/0