Structure of a Hydroxyl Radical Induced DNA–Protein Cross-Link Involving Thymine and Tyrosine in Nucleohistone†

Miral Dizdaroglu,*,‡ Ewa Gajewski,‡ Prasad Reddy,‡ and Sam A. Margolis§

Center for Chemical Technology and Center for Analytical Chemistry, National Institute of Standards and Technology, Gaithersburg, Maryland 20899

Received October 4, 1988; Revised Manuscript Received December 22, 1988

ABSTRACT: Hydroxyl radical induced formation of a DNA–protein cross-link involving thymine and tyrosine in nucleohistone is described. Hydroxyl radicals were generated in N_2O-saturated aqueous solution by ionizing radiation. Samples of nucleohistone were hydrolyzed with HCl and trimethylsilylated. Analysis of irradiated samples by gas chromatography–mass spectrometry with selected-ion monitoring showed the presence of a thymine–tyrosine cross-link on the basis of typical fragment ions from the previously known mass spectrum of its trimethylsilyl derivative. The yield of this DNA–protein cross-link in nucleohistone was measured at incrementing doses of radiation and found to be a linear function of radiation dose between 14 and 300 Gy ($J \cdot kg^{-1}$). This yield amounted to 0.003 $\mu mol \cdot J^{-1}$. The mechanism of formation of this DNA–protein cross-link is thought to result from H atom abstraction by hydroxyl radicals from the methyl group of thymine followed by the addition of the resultant thymine radical to the carbon 3 position of the tyrosine ring and subsequent oxidation of the adduct radical.

Ionizing and UV radiations and a variety of chemicals are known to produce DNA–protein cross-links in living cells (Smith, 1976; Yamamoto, 1976; Fornace & Little, 1979; Oleinick et al., 1986). Evidence indicates that the bonds formed between DNA and proteins are of a covalent nature (Oleinick et al., 1986; Mee & Adelstein, 1981; Cress & Bowden, 1983). Hydroxyl (OH) radicals produced from water by ionizing radiation appeared to be responsible for the formation of ionizing radiation induced DNA–protein cross-links in isolated chromatin and in intact cells (Oleinick et al., 1986; Mee & Adelstein, 1981). The involvement of histones of the nucleosomal core as well as non-histone proteins in the formation of DNA–protein cross-links has been shown in isolated chromatin exposed to ionizing radiation (Mee & Adelstein, 1981; Olinski et al., 1981). Despite the great effort that has been spent on the study of this type of DNA damage, nothing is known about the chemical nature of DNA–protein cross-links in living cells. Elucidation of the chemical nature of these cross-links is necessary for an understanding of the biological consequences of DNA–protein cross-links in living cells.

Recently, OH radical induced cross-linking of thymine (Thy)[1] to Tyr has been studied in a model system, i.e., an aqueous mixture of Thy and Tyr. The exact chemical structure of an OH radical induced Thy–Tyr cross-link has been elucidated by the combined use of various analytical techniques including high-performance liquid chromatography, gas chromatography–mass spectrometry (GC–MS), proton and carbon-13 nuclear magnetic resonance spectroscopy, and high-resolution mass spectrometry (Margolis et al., 1988). The structure of the Thy–Try cross-link has been identified as the product from the formation of a covalent bond between the methyl group of Thy and carbon 3 of the Tyr ring. Here, we present the evidence for the formation of this Thy–Tyr cross-link in calf thymus nucleohistone exposed to ionizing radiation in N_2O-saturated buffered aqueous solution.

Experimental Procedures

Materials.[2] Calf thymus nucleohistone, thymine, tyrosine, protein assay Kit P 5656, and phenylalanylphenylalanine (Phe-Phe) were purchased from Sigma Chemical Co. Histones H2A, H2B, and H3 were from Boehringer Mannheim. Bis(trimethylsilyl)trifluoroacetamide (BSTFA), acetonitrile, and 6 M HCl were obtained from Pierce Chemical Co. Dialysis membranes with a molecular weight cutoff of 3500 were purchased from Fisher Scientific Co. Reagents for electrophoresis were obtained from Bethesda Research Laboratories.

Irradiations. Solutions of calf thymus nucleohistone (0.35 mg/mL) in 10 mM phosphate buffer (pH 7.0) were saturated with N_2O and irradiated in a ^{60}Co γ-source at doses ranging from 14 to 300 Gy ($J \cdot kg^{-1}$). The dose rate of the ^{60}Co γ-source (140 Gy/min) was determined by Fricke dosimetry (Fricke & Hart, 1966). After irradiation, nucleohistone solutions were dialyzed extensively against water and then lyophilized.

Hydrolysis with Hydrochloric Acid and Trimethylsilylation. Aliquots (2.5 mg) of lyophilized samples were hydrolyzed with 1 mL of 6 M HCl in evacuated and sealed tubes for 6 h at 120 °C. Samples were lyophilized and then trimethylsilylated in poly(tetrafluoroethylene)-capped hypovials (Pierce) with 0.25 mL of a BSTFA/acetonitrile (1.5:1 v/v) mixture by heating for 30 min at 130 °C.

Gas Chromatography–Mass Spectrometry (GC–MS). Analysis of samples was performed by using a mass-selective detector interfaced to a gas chromatograph (both from Hew-

† This work has been supported in part by the Armed Forces Radiobiology Research Institute, Bethesda, MD, under Research Work Unit B5103. The views expressed in this article are those of the authors and do not reflect the official views or position of the Defense Department or the U.S. Government.

* Author to whom correspondence should be addressed.
‡ Center for Chemical Technology.
§ Center for Analytical Chemistry.

[1] Abbreviations: Thy, thymine; GC–MS, gas chromatography–mass spectrometry; Phe-Phe, phenylalanylphenylalanine; BSTFA, bis(trimethylsilyl)trifluoroacetamide; Gy, gray ($J \cdot kg^{-1}$); SIM, selected-ion monitoring; RMRF, relative molar response factor.

[2] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

0006-2960/89/0428-3625$01.50/0

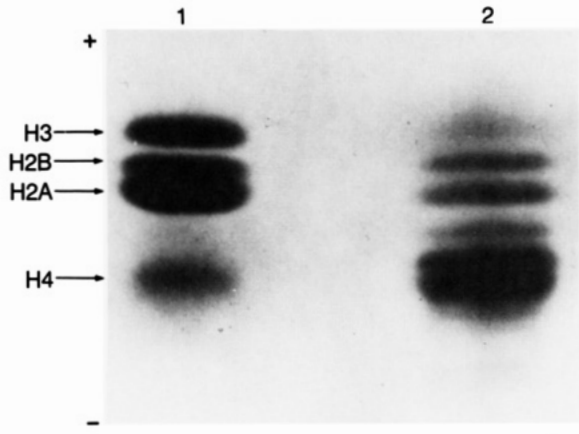


FIGURE 1: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of histones H3, H2B, and H2A (lane 1) and calf thymus nucleohistone (lane 2).

lett-Packard). The injection port, the ion source, and the interface were maintained at 250 °C. Separations were carried out by using a fused silica capillary column (12.5 m × 0.32 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness 0.17 μm) (Hewlett-Packard). Helium was used as the carrier gas at an inlet pressure of 5 kPa. Mass spectra were obtained at 70 eV. The split mode was used for injections.

Gel Electrophoresis. The protein components of the nucleohistone were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis essentially as described by Laemmli (1970) with a modification in the ratio of acrylamide to bis(acrylamide). A Hoefer SE 600 slab gel electrophoresis unit was used. Separating gel (18%) and stacking gel (4%) were prepared from a stock solution of 29.8% acrylamide and 0.2% bis(acrylamide). Electrophoresis was carried out in a 15 × 18 cm slab gel at 40 mA for 3–4 h. Gels were stained with Coomassie blue. The DNA content of nucleohistone was determined by measurement of UV absorption at 260 nm (Marmur & Doty, 1962). The protein content of nucleohistone was measured according to the Lowry procedure (Peterson, 1977).

Results

The objective of this investigation was to determine whether a Thy–Tyr cross-link, which has been found in a γ-irradiated mixture of Thy and Tyr in a previous work (Margolis et al., 1988), was formed in a DNA–protein complex exposed to ionizing radiation in aqueous solution. For this purpose, calf thymus nucleohistone was used. This commercial preparation contained 39% DNA, 46% protein, and 15% unidentified material (by weight). The authenticity of the protein components of this nucleohistone was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using commercially available histones as reference compounds. Figure 1 illustrates gel electrophoretic patterns of reference compounds (lane 1) and the nucleohistone (lane 2). Individual runs of commercial histones showed that histones H2A and H3 were electrophoretically pure. However, histone H2B contained another protein, which presumably corresponded to histone H4. This assumption was based on the molecular weight and the electrophoretic behavior of this histone known from published data. The electrophoretic pattern of the histones H3, H2B, H2A, and H4 illustrated in lane 1 in Figure 1 is quite similar to those published previously (Panyim & Chalkley, 1969; Böhm et al., 1973; Mee & Adelstein, 1981). The nucleohistone used in this work (lane 2 in Figure 1) appeared to contain histones H2B, H2A, and H4 and other proteins. A weak band corresponding to histone H3 was also observed.

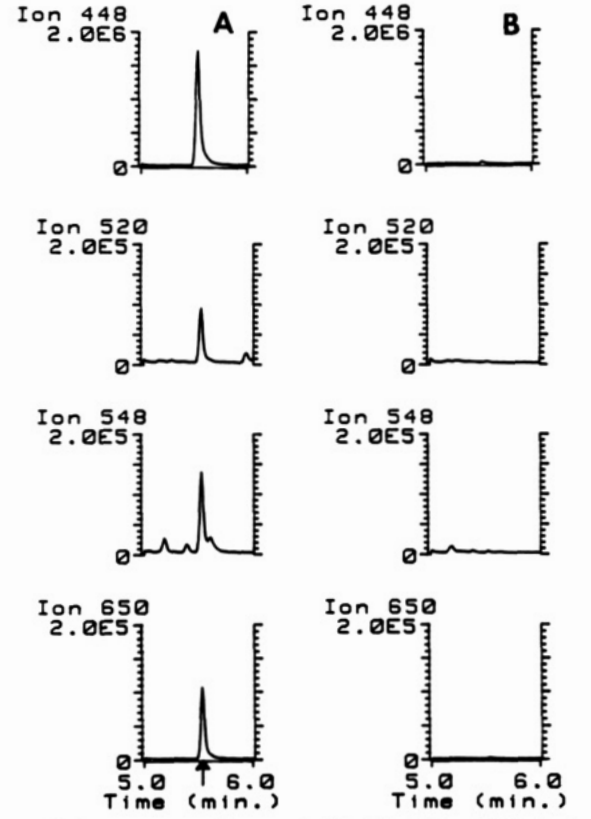


FIGURE 2: Ion-current profiles at m/z 448, 520, 548, and 650 obtained during GC–MS/SIM analysis of trimethylsilylated hydrolysates of nucleohistone. (A) γ-Irradiated in N_2O-saturated aqueous solution at a dose of 300 Gy; (B) nonirradiated. The column was programmed from 200 to 270 °C at a rate of 10 °C/min after 1 min at 200 °C. For other details see Experimental Procedures. It should be noted that the full scales of the m/z 520, 548, and 650 ions are 10 times that of the m/z 448 ion.

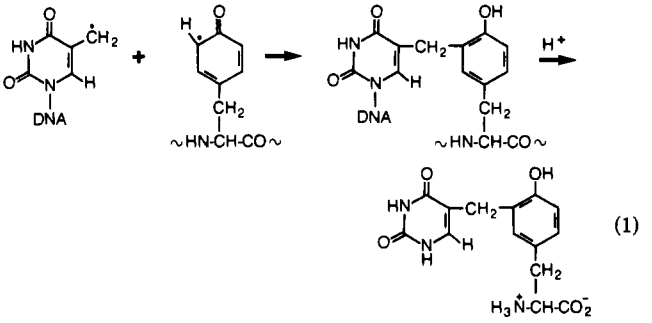
For identification of the Thy–Tyr cross-link, nucleohistone was γ-irradiated in N_2O-saturated buffered aqueous solution, hydrolyzed with 6 M HCl, and then trimethylsilylated and analyzed by GC–MS with selected-ion monitoring (SIM). A number of characteristic ions from the known mass spectrum of the trimethylsilyl (Me_3Si) derivative of the Thy–Tyr cross-link (Margolis et al., 1988) were monitored during the GC–MS/SIM analysis. Nonirradiated samples of nucleohistone were prepared and analyzed in the same manner. Parts A and B of Figure 2 illustrate the ion-current profiles at m/z 448, 520, 548, and 650 obtained during the GC–MS/SIM analysis of irradiated and nonirradiated samples, respectively. Eight characteristic ions were monitored in the same time interval (between 5 and 6 min); however, for practical reasons, only profiles of four ions are illustrated in Figure 2. The readily discernible peaks of the monitored ions are seen at the expected retention time (indicated with an arrow in Figure 2A) of the Me_3Si derivative of the Thy–Tyr cross-link. A partial mass spectrum was obtained subsequently, on the basis of the monitored ions and their abundances, because the relative abundances of the monitored ions must also match those of the same ions in the mass spectrum of the authentic compound for an unequivocal identification. In terms of the monitored ions and their abundances, this partial mass spectrum was identical with the mass spectrum of the Me_3Si derivative of the authentic Thy–Tyr cross-link (data not shown here), meaning the unequivocal identification of this cross-link in γ-irradiated nucleohistone. The mass spectrum of the Me_3Si

derivative of the Thy–Tyr cross-link has been published previously (Margolis et al., 1988). As can be seen from the profile of the m/z 448 ion in Figure 2B, small but detectable amounts of this cross-link were also observed in nonirradiated nucleohistone. The origin of the Thy–Tyr cross-link in nonirradiated nucleohistone is not known.

The quantitative measurement of the Thy–Tyr cross-link in nucleohistone γ-irradiated to doses of 14–300 Gy was carried out by using the GC–MS/SIM technique. For this purpose, a dipeptide (Phe-Phe) was used as an internal standard. After irradiation, dialysis, and 6 M HCl hydrolysis, an aliquot of the internal standard was added to an aliquot of the nucleohistone sample. Following the addition of the internal standard, the sample was frozen immediately in liquid nitrogen and then lyophilized. This was followed by trimethylsilylation and GC–MS/SIM analysis. Ion currents of the m/z 448 ion from the mass spectrum of the Me_3Si derivative of the Thy–Tyr cross-link (Margolis et al., 1988) and of the most intense ion (m/z 192) from the mass spectrum of the Me_3Si derivative of Phe-Phe (Krutzsch, 1983) were measured during the GC–MS/SIM analysis. When the GC–MS/SIM technique is used for quantitative measurements, the mass spectrometer is usually calibrated with samples containing known amounts of the analyte and the internal standard (Watson, 1985). Prior to quantitative analysis, the molar response factor[3] for a characteristic and intense ion of the analyte relative to a characteristic ion of the internal standard is obtained. However, due to the lack of sufficient amount of the authentic material, the relative molar response factor could not be determined experimentally for the Thy–Tyr cross-link reported here. Instead, an estimate of the relative molar response factor was made by comparing the relative abundance of the m/z 448 ion in the mass spectrum of the Me_3Si derivative of the Thy–Tyr cross-link to the relative abundance of the m/z 192 ion in the mass spectrum of the Me_3Si derivative of Phe-Phe. For this purpose, the mass spectra of these compounds were recorded under the same tuning conditions of the mass spectrometer. By use of the estimated relative molar response factor, which amounted to 1.6, the yield of the Thy–Tyr cross-link in nucleohistone was measured by GC–MS/SIM and was found to be a linear function of radiation dose in the dose range studied (14–300 Gy). The "estimated" G value (yield per 1 J of radiation energy), which was calculated from the linear dose–yield plot, amounted to 0.003 ± 0.0002 $\mu mol \cdot J^{-1}$. This value represents approximately 0.5% of the total OH radical yield in an aqueous system saturated with N_2O [0.56 $\mu mol \cdot J^{-1}$ (von Sonntag, 1987)]. This means that approximately 0.5% of OH radicals produced by ionizing radiation from water in this system caused formation of the Thy–Tyr cross-link in nucleohistone.

Discussion

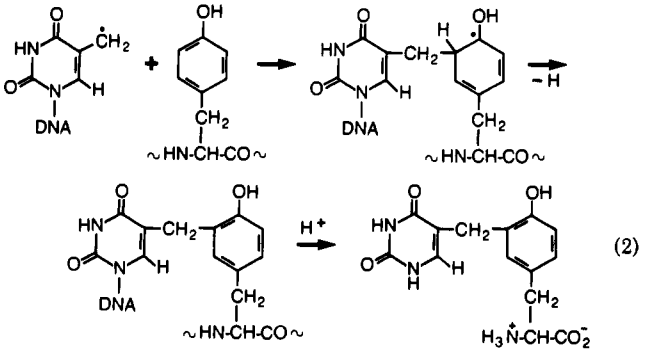
The evidence presented in the present paper indicates that the cross-linking of Thy to Tyr occurs in a DNA–protein complex such as nucleohistone when it is exposed to γ-irradiation in N_2O-saturated buffered aqueous solution. The exposure of dilute aqueous solutions to ionizing radiation causes the formation of OH radicals, H atoms, and hydrated electrons from water [for a review see von Sonntag (1987)]. When N_2O is present in solution, hydrated electrons react with N_2O in a diffusion-controlled reaction to give additional OH radicals. The system then consists of 90% OH radicals and 10% H atoms in terms of radical species. Reactions of OH radicals with nucleohistone are expected to produce radicals on DNA and on histones. Hydroxyl radicals react with DNA bases and aromatic amino acids predominantly by addition to the double bonds of these molecules [for a review see von Sonntag (1987)]. In the case of Thy, the major site of attack is the 5,6 double bond; the abstraction of an H atom from the methyl group of Thy also occurs to a lesser extent (Fujita & Steenken, 1981). Addition of OH radicals to the aromatic ring of Tyr gives rise to dihydroxycyclohexadienyl radicals (Dorfman et al., 1962). The most prominent adduct radical, which is formed by addition of OH radical to the C-3 of Tyr, eliminates water to give a phenoxyl radical (Land & Ebert, 1967). The formation of the Thy–Tyr cross-link in nucleohistone identified in the present work can be explained by either combination of two radicals or addition of a radical to another molecule. In the first case, Thy and Tyr radicals formed by OH radical reactions in nucleohistone may undergo a combination reaction to give the Thy–Tyr cross-link identified in this work as illustrated:



(1)

Cross-linking via combination of a DNA radical and a protein radical in nucleohistone requires the formation of two radicals in close proximity because of the impaired mobility of the macromolecules involved. At low radiation doses used in this work, the OH radical mediated formation of two radicals in close proximity appears to be an unlikely process.

In the case of radical addition reaction, the Thy–Tyr cross-link identified here might be formed by addition of the Thy radical (see reaction 1) to the C-3 position of the Tyr ring and the subsequent oxidation of the so-formed adduct radical as illustrated:



(2)

This mechanism requires the close proximity of the Thy radical to a Tyr molecule in the DNA–protein complex. Recently, the possible formation of a unique hydrogen bond between the hydroxyl group of Tyr and the oxygen at the C-4 position of a neighboring Thy has been described (Hendry et al., 1981). This might permit the close proximity of the methyl group of Thy to the C-3 position of Tyr. The final product of reaction 1 is the same as that of reaction 2. Thus the two mechanisms illustrated above cannot be distinguished from each other by their final product. For the reasons outlined above, however,

[3] The definition of a relative molar response factor (RMRF) is as follows: RMRF = (amount of the analyte)/(amount of the standard) × (peak area of the ion of the standard)/(peak area of the ion of the analyte).

reaction 2 appears to be the likely mechanism for the formation of the Thy–Tyr cross-link in nucleohistone identified in this work.

When oxygen is present in the system, the Thy–Tyr cross-link in nucleohistone is not expected to be formed because diffusion-controlled reactions of oxygen with pyrimidine (or amino acid) radicals convert them into peroxyl radicals inhibiting combination or addition reactions of radicals [for a review see von Sonntag (1987)].

In conclusion, the OH radical induced formation of a DNA–protein cross-link between the methyl group of a Thy moiety and the C-3 position of a Tyr moiety in calf thymus nucleohistone in aqueous solution was described. The methodology used here might permit the study of the DNA–protein cross-links induced by ionizing radiation or other free radical processes in living cells.

Registry No. Hydroxyl, 3352-57-6; thymine, 65-71-4; tyrosine, 60-18-4; 3-[(1,3-dihydro-2,4-dioxopyrimidin-5-yl)methyl]-L-tyrosine, 118949-95-4.

References

Böhm, E. L., Strickland, W. N., Strickland, M., Thwaits, B. H., van der Westhuizen, D. R., & von Holt, C. (1973) *FEBS Lett. 34*, 217–221.

Cress, A. E., & Bowden, G. T. (1983) *Radiat. Res. 95*, 610–618.

Dorfman, L. M., Taub, I. A., & Bühler, R. E. (1962) *J. Chem. Phys. 36*, 3051–3061.

Fornace, A. J., Jr., & Little, J. B. (1979) *Cancer Res. 39*, 704–710.

Fricke, H., & Hart, E. J. (1966) in *Radiation Dosimetry* (Attix, F. H., & Roesch, W. C., Eds.) Vol. II, pp 167–239, Academic Press, New York.

Fujita, S., & Steenken, S. (1981) *J. Am. Chem. Soc. 103*, 2540–2545.

Hendry, L. B., Bransome, E. D., Jr., Hutson, M. S., & Campbell, L. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7440–7444.

Krutzsch, H. C. (1983) *Methods Enzymol. 91*, 511–524.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Land, E. J., & Ebert, M. (1967) *Trans. Faraday Soc. 63*, 1181–1190.

Margolis, S. A., Coxon, B., Gajewski, E., & Dizdaroglu, M. (1988) *Biochemistry 27*, 6353–6359.

Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 5*, 109–118.

Mee, L. K., & Adelstein, S. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2194–2198.

Oleinick, N. L., Chiu, S., Friedman, L. R., Xue, L., & Ramakrishnan, N. (1986) in *Mechanisms of DNA Damage and Repair, Implications for Carcinogenesis and Risk Assessment* (Simic, M. G., Grossman, L., & Upton, A. C., Eds.) pp 181–192, Plenum, New York.

Olinski, R., Briggs, R. C., Hnilica, L. S., Stein, J., & Stein, G. (1981) *Radiat Res., 86*, 102–114.

Panyim, S., & Chalkley, R. (1969) *Arch. Biochem. Biophys. 130*, 337–346.

Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.

Smith, K. C. (1976) in *Aging, Carcinogenesis and Radiation Biology* (Smith, K. C., Ed.) pp 67–91, Plenum, New York.

von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, London.

Watson, J. T. (1985) *Introduction to Mass Spectrometry*, pp 59–74, Raven Press, New York.

Yamamoto, O. (1976) in *Aging, Carcinogenesis and Radiation Biology* (Smith, K. C., Ed.) pp 165–192, Plenum, New York.

CORRECTIONS

Interactions of Oleic Acid with Liver Fatty Acid Binding Protein: A Carbon-13 NMR Study, by David P. Cistola,* Mary T. Walsh, Ronald P. Corey, James A. Hamilton, and Peter Brecher, Volume 27, Number 2, January 26, 1988, pages 711–717.

Page 716. In column 2, the last two sentences beginning on line 8 should read as follows: Results obtained with fluorescence spectroscopy indicated that anthroyloxy-labeled FA analogues have an affinity for liver FABP molecules an order of magnitude greater than that for phosphatidylcholine molecules (Storch et al., 1986). When the NMR-derived partition coefficients shown in Table I (moles of 18:1 bound per gram of PC) are expressed as moles of 18:1 bound per mole of PC, the results obtained with ^{13}C-enriched FA agreed with those obtained with fluorescent FA analogues.

Stabilization of Microtubules by Inorganic Phosphate and Its Structural Analogues, the Fluoride Complexes of Aluminum and Beryllium, by M. F. Carlier,* D. Didry, R. Melki, M. Chabre, and D. Pantaloni, Volume 27, Number 10, May 17, 1988, pages 3555–3559.

Page 3556. Under Materials and Methods, paragraph 3, lines 7–9 should read as follows: ...diluted 50-fold at time 0 into PG buffers containing 50% sucrose in the place of glycerol, 10 mM MES, and increasing concentrations, in the range 0–150 mM, of inorganic phosphate.... We acknowledge the help of Dr. Michaël Caplow in bringing this correction to our attention.

Antithrombin III Utah: Proline-407 to Leucine Mutation in a Highly Conserved Region near the Inhibitor Reactive Site, by Susan Clark Bock,* Jean A. Marrinan, and Elzbieta Radziejewska, Volume 27, Number 16, August 9, 1988, pages 6171–6178.

Page 6175. Several C's and T's are reversed at the bottom of Table I where sequence differences for exon 6, position 105, are reported. The antithrombin III (ATIII) Utah allele contains a T at this position, as do the normal ATIII genes referenced in footnotes *b*, *i*, *f*, and *j*. The normal ATIII gene referenced in footnote *h* also contains a T at position 105, although the original report listed a C. Thus, footnote *c* should read as follows: reanalysis of original clone indicates that this nucleotide is a T.

Hydrogen-1 NMR Evidence for Three Interconverting Forms of Staphylococcal Nuclease: Effects of Mutations and Solution Conditions on Their Distribution, by Andrei T. Alexandrescu, Eldon L. Ulrich, and John L. Markley*, Volume 28, Number 1, January 10, 1989, pages 204–211.

Page 205. In column 1, line 6, KO_2H should read KO^2H.

Page 209. In Table I, row 9, N″ (%) should read 0.