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Glucose Oxidase Contains a Disubstituted Phosphorus Residue. Phosphorus-31 Nuclear Magnetic Resonance Studies of the Flavin and Nonflavin Phosphate Residues†

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ABSTRACT: Glucose oxidase from *Aspergillus niger* was studied by ^{31}P NMR. In addition to the two resonances from its flavin adenine dinucleotide (FAD) coenzyme, a resonance from a phosphorus residue covalently bound to the enzyme was also observed. The spectra indicate that the two subunits of glucose oxidase are identical with regard to the single FAD per subunit. Comparison of the ^{31}P NMR spectra of free FAD with that of native glucose oxidase reveals a substantial shift in one of the resonances of the pyrophosphate linkage upon binding to the enzyme. No changes in conformation are indicated for either the FAD pyrophosphate or the covalent phosphorus with change in oxidation state of the flavin ring of FAD among oxidized, semiquinone, and fully reduced species. Formation of a sulfite adduct also leaves the resonances unaltered. The unpaired electron on the FAD in the semiquinone form results in differential broadening of the ^{31}P NMR resonances of the FAD pyrophosphate, indicating that one phosphorus nucleus is nearer to the flavin ring than the other. A lack of broadening in the ^{31}P NMR resonance of the covalent phosphorus for the semiquinone species of glucose oxidase indicates that the covalent phosphorus is remote from the flavin. Addition of paramagnetic Mn(II) to the enzyme solution results in broadening of the ^{31}P NMR resonance assigned to the covalent phosphorus residue, showing that it is near the surface of the enzyme. Mn(II) addition does not perceptibly broaden the ^{31}P NMR resonances of the bound FAD, demonstrating that the pyrophosphate moiety if buried in the enzyme. Glucose oxidase is a glycoprotein. Evidence that the covalent phosphorus moiety is bound to the protein, and not to the carbohydrate moiety, is due to (a) no phosphorus release from the enzyme with periodate treatment and (b) separation of all glycopeptides from the phosphopeptide on a Sephadex G-25 column following Pronase digestion of the enzyme. The observed independence of the ^{31}P NMR chemical shift of the covalently bound phosphorus in the holoprotein and in the phosphopeptide with change in pH from 4.7 to 8.2 shows it to be disubstituted. The implication is that a phospho bridge exists, linking two amino acid residues in the polypeptide chain of the enzyme much as disulfide links in proteins modify the secondary structure.

Glucose oxidase from *Aspergillus niger* is a dimeric enzyme which catalyzes the reaction:

$$\text{D-glucose} + O_2 \rightarrow \text{D-gluconic acid} + H_2O_2$$

Each subunit of the enzyme is ~70 000 daltons and contains ~16% (w/w) carbohydrate as well as 1 mol of firmly bound FAD[1] (Pazur, 1966).

In a previous report (Edmondson & James, 1979), we noted that several flavoproteins contain covalently bound phosphate residues, in addition to those of the flavin coenzyme. Glucose oxidase contains 1 mol of noncoenzyme phosphorus/subunit (Swoboda & Massey, 1966). We have previously demonstrated by using ^{31}P NMR that a covalently bound phosphate in flavodoxin from *Azotobacter vinelandii* is disubstituted, apparently forming a phosphodiester linkage between two hydroxyl amino acids in the protein (Edmondson & James, 1979). The present investigation is designed to provide information on the structure and environment of the covalent phosphorus residue as well as the flavin phosphates and to monitor any alterations of the environment that may occur on changes in oxidation–reduction level of the enzyme.

As demonstrated in this paper by using ^{31}P NMR, the covalently bound phosphorus residue in glucose oxidase is a disubstituted phosphate attached to the protein moiety near the protein surface but remote from the flavin. ^{31}P NMR results on the FAD coenzyme also show that binding of FAD to glucose oxidase changes the conformation of the pyrophosphate moiety. When bound to the enzyme, the pyrophosphate moiety is not exposed on the surface of the enzyme.

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[1] Abbreviations used: FAD, flavin adenine dinucleotide; AMP, adenosine 5′-phosphate; FMN, flavin mononucleotide; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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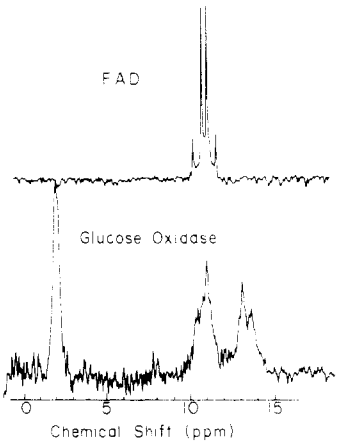

FIGURE 1: 40.5-MHz ^{31}P NMR spectra of (top) 10 mM FAD in 0.1 M Tris–acetate buffer at pH 8.0 and (bottom) 1.7 mM glucose oxidase in 0.1 M Tris–acetate buffer at pH 8.0. Both spectra were obtained with a pulse recycle time of 1.0 s and exponential broadening equivalent to 2 Hz. The number of transients acquired were 252 (top) and 1.1 $\times 10^5$ (bottom).

^{31}P NMR experiments also suggest that the phosphate in the AMP half of the bound coenzyme is nearer to the flavin ring than the phosphate from the FMN half.

Materials and Methods

Glucose oxidase was purchased from Miles Laboratories, Elkhart, IN. After concentration by ultrafiltration, the enzyme preparation was further treated by chromatography on Sephadex G-100 in 0.1 M Tris–acetate, pH 8.0. The different lot numbers for the various enzyme preparations used revealed a single band on $NaDodSO_4$ gel electrophoresis (Weber & Osborne, 1969) and showed identical phosphorus to flavin ratios, as well as carbohydrate content.

The apoenzyme and Pronase-degraded forms of the apoenzyme were prepared as described by Swoboda & Massey (1966). Carbohydrate analyses were performed by the orcinol–sulfuric acid method (Ashwell, 1957), protein concentrations were estimated by the biuret method (Gornall et al., 1949), and phosphorus concentrations were determined by the procedure outlined by Bartlett (1959). FAD was purchased from the Sigma Chemical Co. and used without further purification. Prior to NMR spectral measurements, all solutions were freed of any contaminating paramagnetic impurities by chromatography on a small Chelex column.

^{31}P NMR spectra were obtained at 40.5 MHz on a Varian XL-100-15 spectrometer equipped with a Nicolet Fourier-transform accessory and a multiple other nuclei accessory (MONA). Quadrature phase detection was employed and field–frequency locking was implemented by using the deuterium resonance of the D_2O in the sample. All chemical shifts were determined relative to an external standard of 85% phosphoric acid. All spectra were obtained at 25 ± 3 °C with the samples in 12-mm precision NMR tubes (Wilmad 514A-7PP). Spectra of the semiquinone and fully reduced forms of glucose oxidase were obtained by using anaerobic NMR tubes (Wilmad 514A-7-SJ) with an atmosphere of oxygen-free argon over the samples. All experiments were performed in duplicate or triplicate with different samples.

Results

^{31}P NMR of FAD and Glucose Oxidase. The ^{31}P NMR spectrum of glucose oxidase (in the oxidized state) at pH 8.0 is compared with the spectrum of FAD at the same pH in Figure 1. The same spectrum is obtained (not shown) if the enzyme solution is buffered at pH 5.0. The additional resonance at 2.0 ppm in the spectrum of the enzyme confirms the chemical observation of Swoboda & Massey (1966) that glucose oxidase contains a phosphorus residue in addition to those in the pyrophosphate moiety of the coenzyme.

Treatment of the heat-denatured enzyme with 5% (w/v) trichloroacetic acid precipitates the protein with liberation of FAD. After extensive washing of the precipitate, 1 mol of phosphorus/subunit molecular weight is found. This phosphorus residue is judged, therefore, to be covalently bound.

The ^{31}P NMR spectrum of glucose oxidase in Figure 1 (and in subsequent figures) indicates that the two subunits of the enzyme are identical in that a single set of resonances is observed for the covalent phosphorus and the phosphorus nuclei in the bound FAD.

The ^{31}P NMR spectrum of free FAD in Figure 1 displays an AB splitting pattern. Kainosho & Kyogoku (1972) have previously published the ^{31}P NMR spectrum of FAD, assigning the upfield resonance at 11.3 ppm to the phosphorus nucleus of the AMP moiety and the downfield resonance at 10.8 ppm to the phosphorus nucleus of the FMN moiety of the coenzyme. It would appear from the spectrum of glucose oxidase in Figure 1 that the conformation of the pyrophosphate linkage is altered when FAD is bound to the enzyme; the chemical shift of the downfield resonance is unchanged, but the upfield resonance is now at higher field and the splitting approaches an AX rather than an AB pattern.

Effect of Reduction of the Coenzyme FAD. The FAD coenzyme of glucose oxidase can be reduced by one electron to form the paramagnetic anionic semiquinone (at alkaline pH) and by two electrons to form the diamagnetic hydroquinone. The ^{31}P NMR spectra of glucose oxidase with the FAD in three different oxidation forms at pH 8 are compared in Figure 2. The chemical shifts of the covalent phosphorus resonance and the FAD resonances remain unchanged with variation in oxidation–reduction level or upon formation of a flavin–sulfite adduct.

The electron spin relaxation time of the unpaired electron in the flavin semiquinone is $\sim 10^{-8}$ s (Palmer et al., 1971), which is sufficient to produce measurable line broadening ($\gtrsim 5$ Hz) in a phosphorus resonance if a phosphorus nucleus is within 10 Å of the unpaired electron, according to calculations using the Solomon–Bloombergen equation (James, 1975). It is evident from Figure 2 that the covalent phosphorus residue must be removed from the immediate vicinity of the flavin ring on the enzyme, since the resonance at 2.0 ppm is not broadened perceptibly on semiquinone formation. In contrast, some broadening is apparent in the FAD pyrophosphate resonances for the semiquinone form. In fact, the upfield resonance is broadened to a greater extent than the downfield pyrophosphate resonance. On the basis of the resonance assignments of Kainosho & Kyogoku (1972), it would appear that the AMP phosphate is closer than the FMN phosphate to the flavin ring of FAD in glucose oxidase. Construction of a molecular model indicates that such a configuration is feasible.

It should be noted that there is some difficulty in obtaining a solution containing 100% of the semiquinone form and maintaining that form over the period of time necessary to obtain the ^{31}P NMR spectrum, ~7 h. Consequently, the spectrum attributed to the semiquinone in Figure 2 also contains a small contribution (estimated to be <15%) from the oxidized species. Spectra (not shown) of solutions containing variable amounts of the semiquinone form and either the oxidized or fully reduced form indicate that the spectra are superimpositions of the spectrum due to each form; conse-

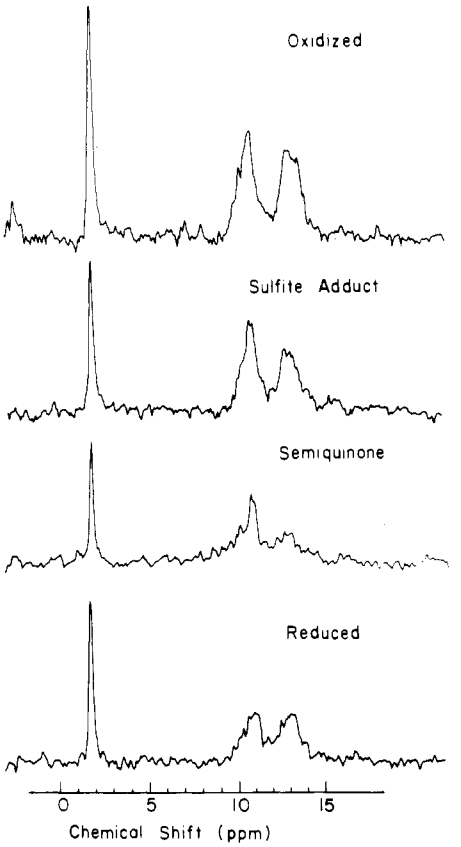

FIGURE 2: Effect of FAD reduction and sulfite adduct formation on the 40.5-MHz ^{31}P NMR spectrum (proton decoupled) of glucose oxidase in 0.1 M Tris–acetate, pH 8.0–8.2. (Top) Oxidized glucose oxidase (2 mM), 1.0-s pulse repetition time, 3-Hz line broadening, 68 000 transients; (second from top) sulfite adduct of glucose oxidase (2 mM), 0.64-s pulse repetition time, 3-Hz line broadening, 84 000 transients; (second from bottom) semiquinone form of glucose oxidase (2 mM), 0.51-s pulse repetition time, 3-Hz line broadening, 46 000 transients; (bottom) hydroquinone form of glucose oxidase (3 mM), 1.0-s pulse repetition time, 3-Hz line broadening, 37 000 transients.

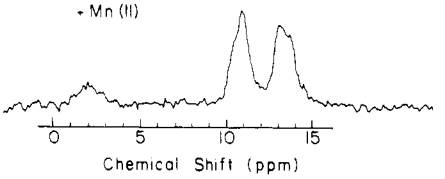

FIGURE 3: Influence of 0.1 mM Mn(II) on the proton-decoupled ^{31}P NMR spectrum of glucose oxidase (2 mM oxidized form) in 0.1 M Tris–acetate, pH 8.0. Pulse repetition time = 1.0 s, line broadening = 3 Hz, and number of transients = 91 000.

quently, there is slow electron exchange between the different oxidation state species on the NMR time scale (<50 s^{-1}) in agreement with the finding of a kinetic flavin radical stabilization by Stankovich et al. (1978) using potentiometric techniques.

Effect of Manganese Ion Addition. Addition of a paramagnetic ion, such as Mn(II), can cause broadening of resonance due to nuclei which are near the Mn(II) in solution (James, 1975). For example, addition of Mn(II) has been used to distinguish solvent-accessible from solvent-inaccessible phospholipids in membrane systems (Michaelson et al., 1974). The ^{31}P NMR spectrum of oxidized glucose oxidase shown in Figure 3 reveals considerable broadening of the resonance due to the covalent phosphorus residue at 2.0 ppm when 0.1 mM Mn(II) is added to the enzyme solution. This experiment shows that the covalent phosphorus must be located near the surface of the enzyme for the resonance to be broadened by the substoichiometric amount (molar ratio of 1:20) of Mn(II) in solution.

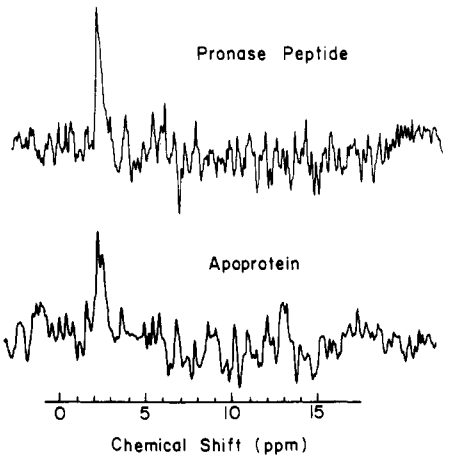

FIGURE 4: Proton-decoupled ^{31}P NMR spectra of (bottom) the glucose oxidase apoprotein (1 mM), after removal of FAD, in 3 M guanidine hydrochloride 0.1 M and Tris–acetate, pH 8.5, obtained with 1.0-s pulse repetition time, 5-Hz line broadening, and 63 000 acquisitions, and (top) the phosphopeptide obtained after Pronase digestion and isolation on a Sephadex G-25 column. The phosphopeptide (1 mM) was in 0.1 M Tris–acetate buffer, pH 4.7. Spectral parameters were 1.0-s pulse recycle time, 3-Hz line broadening, and 92 000 acquisitions.

In contrast, the spectrum in Figure 3 reveals very little broadening of the ^{31}P NMR resonances from the bound FAD. It must be concluded that the pyrophosphate moiety of the coenzyme is "buried" within the enzyme at a considerable distance from the surface.

^{31}P NMR Spectra of the Apoprotein and the Phosphorus-Containing Peptide. Sephadex chromatography at pH 1.5 resolves the FAD coenzyme from the apoprotein (Swoboda & Massey, 1966). The ^{31}P NMR spectrum of the apoprotein in 3 M guanidine hydrochloride at pH 8.5 is shown in Figure 4. The low solubility of the apoprotein results in the spectrum with low signal to noise ratio. The spectrum of the apoprotein reveals one resonance at 2.3 ppm which is shifted only slightly upfield of the holoprotein resonance (2.0 ppm).

Treatment of the apoprotein with periodate is known to remove ~40% of the carbohydrate moiety of glucose oxidase (Nakamura et al., 1976). Such treatment, however, did not result in the release of phosphorus from the protein moiety. The apoprotein was also digested with Pronase, and the resultant phosphorus-containing fraction was isolated after chromatography over a Sephadex G-25 column. The glycopeptide fraction accounting for >90% of the carbohydrate eluted in the excluded volume of the column. No phosphorus could be detected in this fraction. The phosphopeptide eluted in the included volume of the column with no detectable carbohydrate content. Peptides other than the phosphopeptide were present in this fraction, however. The ^{31}P NMR spectrum of the phosphopeptide at pH 4.7 is shown in Figure 4. The chemical shift of the resonance did not change when the pH of the phosphopeptide solution was raised to 8.2; the chemical shift of 2.2 ppm is virtually the same as that of the apoprotein. These data, as well as data for the holoenzyme, show that the covalent phosphorus residue is incapable of ionization over this pH range. Phosphoproteins and model compounds (including phosphoesters, acyl phosphates, phosphoimidazole, and phospholysine) with monosubstituted phosphorus residues all shift 2–5 ppm downfield when the pH is lowered over that range (Bock & Sheard, 1975). It is therefore concluded that the covalently bound phosphorus residue in glucose oxidase is disubstituted with amino acid

residues as the likely substituents.

Discussion

Nature of the Covalent Phosphorus Residue. The resonance at 2.0 ppm in the ^{31}P NMR spectra of Figures 1 and 2 indicates that the environment of the phosphorus residue is identical in the two subunits and that changes in oxidation state are also without effect on its environment. The spectrum of glucose oxidase in the flavin semiquinone state (see Figure 2) further shows the covalent phosphorus residue to be removed from the region of the flavin ring. Broadening of the peak upon addition of paramagnetic Mn(II) indicates that the covalent phosphorus is near the surface of the enzyme.

Evidence that the phosphorus is bound to the protein rather than the carbohydrate moiety of glucose oxidase is provided by the following data. Treatment of the apoenzyme with periodate does not result in the release of any phosphate. Since the phosphate residue is located near the surface of the protein, it can be argued that if bound to a sugar moiety, it should be susceptible to periodate cleavage. A more definitive experiment is the finding that complete resolution of the glycopeptide fraction from the phosphopeptide fraction can be achieved by chromatography of a Pronase digest of the apoenzyme on a Sephadex G-25 column.

Evidence that the covalent phosphorus residue is disubstituted is provided primarily by the invariance of the chemical shift with pH for the holoenzyme, the apoprotein, and the phosphopeptide isolated from Pronase-digested enzyme. The chemical shift of 2 ppm provides secondary evidence, being uncharacteristic of dianionic phosphates but in the range of monoanionic phosphate groups.

We have also recently found that flavodoxin from *Azotobacter vinelandii* contains a disubstituted covalent phosphate which has a ^{31}P NMR resonance at −0.9 ppm (Edmondson & James, 1979). It was suggested that *Azotobacter* flavodoxin contained a phosphodiester between two hydroxy amino acid residues of its polypeptide chain. That suggestion has been confirmed by further chemical experiments.[2] Although the results reported in the present paper also suggest that the covalent phosphate in glucose oxidase serves to link two amino acid residues of its polypeptide chain, the chemical shift of 2.0–2.3 ppm indicates that the same two amino acid residues as in flavodoxin probably are not involved. A chemical shift in the range of −0.9 ppm is quite characteristic of phosphate esters of hydroxy amino acids and similar model compounds (Bock & Sheard, 1975). However, a chemical shift of 2 ppm is in the range where acyl phosphates, phospholysine, and phosphoimidazole must also be considered. It has been found that base hydrolysis (0.01 N NaOH, 60 °C, 2 h) releases phosphate from the apoprotein, thus arguing against the presence of a P–N bond. Nevertheless, at the present time, the identity of the two amino acid residues linked by the phosphate bridge remains unknown. Glucose oxidase and *Azotobacter* flavodoxin are, at present, the only proteins demonstrated to contain a phosphate bridge linking two amino acid residues of the polypeptide chain.

Although it is possible that the disubstituted covalent phosphorus residues perform more directly in the biological function of glucose oxidase and *Azotobacter* flavodoxin, a structural role seems likely in that a bridge is being formed between two amino acid residues of the polypeptides. The disubstituted phosphate could stabilize protein secondary structure in a manner similar to that of disulfide bridges in proteins.

FAD Binding to Glucose Oxidase. It is apparent from an examination of the spectra in Figures 1 and 2 that the two subunits of glucose oxidase are identical with regard to the pyrophosphate moiety of bound FAD. It is further evident from Figure 2 that change in oxidation state or formation of the sulfite adduct has negligible influence on the structure of the pyrophosphate linkage in bound FAD.

The experiment involving Mn(II) addition to glucose oxidase results in little broadening of the FAD ^{31}P NMR resonances (see Figure 3). These data suggest strongly that the FAD pyrophosphate is buried in the enzyme and agree with the finding of Nakamura et al. (1976) that the polyhydroxy side chain of the bound FAD is "buried" in the protein since it is not cleaved on periodate treatment.

Comparison in Figure 1 of the ^{31}P NMR spectra of free FAD and FAD tightly bound to the enzyme clearly reveals an alteration when FAD is bound. Gorenstein (1975, 1977) has shown that ^{31}P NMR chemical shifts of phosphate esters are primarily established by the torsional angles and bond angle in the phosphoester; it is not possible to distinguish between the two effects since they are strongly coupled. It would appear from the spectra in Figure 1 that the downfield resonance remains in the same position but the upfield resonance shifts further upfield. Since Kainosho & Kyogoku (1972) have assigned the upfield ^{31}P NMR resonance of FAD to the AMP phosphate nucleus, it would appear that the conformation in that particlar moiety has been altered by binding of the coenzyme to glucose oxidase. The 2-ppm upfield shift of the resonance could result from an increase in the PO–P–OR bond angle and/or a change in the PO–P–OR torsional angle going from a trans to a gauche conformation (Gorenstein, 1977). The possibility cannot be ruled out, however, that the upfield resonance of free FAD coincidentally moves downfield 0.5 ppm and the downfield resonance (attributed by Kainosho & Kyogoku (1972) to the FMN moiety's phosphorus) moves upfield 2.5 ppm upon binding to glucose oxidase; that would also yield the observed ^{31}P NMR spectrum. Specific ^{18}O labeling of one phosphate would be required to resolve these possibilities.

It is interesting to note that the binding of FAD to glucose oxidase is very strong with a dissociation constant of $>10^{-10}$ M. Previous observations of Nageswara Rao & Cohn (1977a) and Nageswara Rao et al. (1979) have indicated little variation in ^{31}P NMR chemical shifts when phosphate-containing substrates and inhibitors bind weakly to enzymes. However, significant ^{31}P NMR chemical shift alterations occur with tight binding of ligands such as phosphoenolpyruvate to pyruvate kinase (Nageswara Rao et al., 1979), MgAppA to adenylate kinase (Nageswara Rao & Cohn, 1977b), and diphosphoglycerate to diphosphoglycerate kinase (Nageswara Rao et al., 1978). Tight binding may result in some alteration of the torsional angles and bond angles of substrates, inhibitors, and coenzymes.

This paper demonstrates the usefulness of ^{31}P NMR spectra for examining the structures and environments of phosphorus nuclei in enzymes. The ^{31}P NMR experiments may be used to probe the nature of covalently bound phosphorus, as well as tightly bound coenzymes. Flavoenzymes are particularly amenable to such studies.

[2] β elimination experiments followed by acid hydrolysis and analysis of the resultant α-keto acid have established the two amino acids linked to the covalent phosphorus in *Azotobacter* flavodoxin as serine and threonine (D. E. Edmondson and T. L. James, unpublished results).

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Structure of Chromatin at Deoxyribonucleic Acid Replication Forks: Location of the First Nucleosomes on Newly Synthesized Simian Virus 40 Deoxyribonucleic Acid†

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ABSTRACT: Exonucleases specific for either 3′ ends (*Escherichia coli* exonuclease III) or 5′ ends (bacteriophage T7 gene 6 exonuclease) of nascent DNA chains have been used to determine the number of nucleotides from the actual sites of DNA synthesis to the first nucleosome on each arm of replication forks in simian virus 40 (SV40) chromosomes labeled with [^{3}H]thymidine in whole cells. Whereas each enzyme excised all of the nascent [^{3}H]DNA from purified replicating SV40 DNA, only a fraction of the [^{3}H]DNA was excised from purified replicating SV40 *chromosomes*. The latter result was attributable to the inability of either exonuclease to digest nucleosomal DNA in native replicating SV40 chromosomes, as demonstrated by the following observations: (i) digestion with either exonuclease did not reduce the amount of newly synthesized nucleosomal DNA released by micrococcal nuclease during a subsequent digestion period; (ii) in briefly labeled molecules, as much as 40% of the [^{3}H]DNA was excised from long nascent DNA chains; (iii) the fraction of [^{3}H]DNA excised by exonuclease III was reduced in proportion to the actual length of the radiolabeled DNA; (iv) the effects of the two exonucleases were additive, consistent with each enzyme trimming only the 3′ or 5′ ends of nascent DNA chains without continued excision through to the opposite end. When the fraction of nascent [^{3}H]DNA excised from replicating SV40 DNA by exonuclease III was compared with the fraction of [^{32}P]DNA simultaneously excised from an SV40 DNA restriction fragment, the actual length of nascent [^{3}H]DNA was calculated. From this number, the fraction of [^{3}H]DNA excised from replicating SV40 *chromosomes* was converted into the number of nucleotides. Accordingly, the average distance from either 3′ or 5′ ends of long nascent DNA chains to the first nucleosome on either arm of replication forks was found to be 125 nucleotides. Furthermore, each exonuclease excised about 80% of the radiolabel in Okazaki fragments, suggesting that less than one-fifth of the Okazaki fragments were contained in nucleosomes. On the basis of these and other results, a model for eukaryotic replication forks is presented in which nucleosomes appear rapidly on both the forward and retrograde arms, about 125 and 300 nucleotides, respectively, from the actual site of DNA synthesis. In addition, it is proposed that Okazaki fragments are initiated on nonnucleosomal DNA and then assembled into nucleosomes, generally after ligation to the 5′ ends of long nascent DNA chains is completed.

Replication of eukaryotic chromosomes requires the accurate duplication of both DNA sequence and chromatin organization. Discrete particles, called "nucleosomes", each containing about 200 base pairs of DNA coiled around an octamer of histones H2A, H2B, H3, and H4, represent the primary level of chromatin organization (Kornberg, 1977; Lilley & Pardon, 1979; McGhee & Felsenfeld, 1980). The invariant portion of a nucleosome, the "core", consists of the histone octamer and 145 base pairs of DNA, while the remainder of the DNA is called the "linker" or "spacer" and consists of 10–100 base pairs of DNA depending upon the source of the chromatin (Thomas & Thompson, 1977; Prunell & Kornberg, 1978; Shelton et al., 1980; McGhee & Felsenfeld, 1980). When nucleosomes are not arranged contiguously, as in SV40[1] chromosomes, spacer DNA can include nonnucleo-

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