A Combined Proton and Phosphorus-31 Nuclear Magnetic Resonance Investigation of the Combining Site of M603, a Phosphocholine-Binding Myeloma Protein†

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ABSTRACT: Phosphorus-31 nuclear magnetic resonance (NMR) studies on the two phosphorus nuclei of the phosphonium analogue ($Me_3P^+CH_2CH_2OPO_3^{2-}$) of phosphocholine are used to monitor the charged subsites in the phosphocholine-binding immunoglobulin A mouse myeloma M603. Comparison of the 270-MHz ^{1}H NMR difference spectrum on addition of either this analogue or phosphocholine to M603 and the almost identical changes in the pK_a values of the phosphate groups on binding to M603 confirm that the analogue is a good model for phosphocholine. The pK_a of the phosphate groups is decreased by 0.5 unit on binding to M603, which is consistent with the phosphate group being hydrogen bonding to Tyr-33$_H$ and Arg-95$_L$, as suggested from the X-ray structure, and also implies that the binding energies for the mono- and dianion are similar. The P^+Me_3 moiety is used to probe the electrostatic interactions in the choline subsite. Titration of the chemical shift of the phosphonium phosphorus reflects a group on the protein that has a pK_a value of ≤5, which from the refined X-ray structure (D. R. Davies, personal communication) of the site is assigned to Asp-97$_L$. The choline substite is monitored by using ^{1}H NMR difference spectra, which indicates that the subsite is highly aromatic as expected from the crystal structure that places Trp-107$_H$ and Tyr-100$_L$ in this subsite. The ring current interactions from these rings can account for the ^{1}H NMR chemical shift data on choline.

The discovery that M603 binds phosphocholine was made by Leon & Young (1971) while they were searching for the determinant responsible for precipitation of certain myeloma proteins with pneumococcal C polysaccharide. Thirteen such myeloma proteins have been found (Potter, 1977), for all of which, phosphocholine inhibits precipitation. In addition, the relative inhibitory abilities of choline and L-α-glycerophosphocholine were investigated. For all but three of the myeloma proteins (M511, M167, and T15), the strength of binding is phosphocholine > L-α-glycerophosphocholine > choline, thereby suggesting the importance of both positive and negative subsites in the antibody combining site for high-affinity binding of hapten (Leon & Young, 1971).

The sequences of eight V_H[1] regions from phosphocholine-binding proteins other than M603 show that all belong to the VH-4 isotype (Rudikoff & Potter, 1974; Hood et al., 1976) with a maximum of five framework substitutions (for M167) compared with T15. Most of the V_L sequences (Barstad et al., 1974; Potter et al., 1976) have been determined only as far as the invariant residue tryptophan-35$_L$ and show three quite distinct isotypes (VK-4, VK-22, and VK-24), though they have a very long L1 hypervariable region in common. Because of this restricted range of V_H and V_L isotypes, attempts were made to determine how myeloma proteins, which can bind phosphocholine, relate to antibodies raised by immunization with a wide range of antigens containing phosphocholine. partial sequence analyses of Rudikoff & Claflin (1976) confirmed the findings of studies with antiidiotype antisera (Claflin & Davie, 1974; Claflin et al., 1974; Cosenza & Köhler, 1972; Sher & Cohn, 1972; Gearhart et al., 1975). These showed that the VK-4, VK-22, and VK-24 isotopes occur in the responses of most conventional mouse stains to phosphocholine, whereas BALB/c shows a preponderance of T15 idiotype (VH-4A and VIC-22) antibodies.

This close connection between phosphocholine-binding myeloma proteins and naturally raised anti-phosphocholine antibodies makes the X-ray crystal structure of M603 (Segal et al., 1974; Padlan et al., 1973, 1976) and the details of hapten binding very important.

The refined X-ray crystal structure (D. R. Davies, personal communication) for the binding site of the M603–phosphocholine complex (Figure 4) indicates that the phosphate group interacts with the protein side chains of Tyr-33$_H$ and Arg-52$_H$. At the other end of the hapten, there is a carboxyl group, Asp-97$_L$, in close proximity to the N^+Me_3 grouping while Trp-107$_H$ and Tyr-100$_L$ are also contact residues for the hapten. This structure is consistent with studies that have shown that a large portion of the binding energy for the interaction between hapten and phosphocholine-specific antibodies arises from electrostatic interactions between oppositely charged groups. This is supported by the findings that partial esterification of the carboxyl group of a related protein H-8 greatly reduced its affinity (Grossberg et al., 1974) and the lack of detectable binding to phosphocholine-binding proteins of any compounds lacking the quaternary nitrogen (Leon & Young, 1971).

^{31}P NMR affords a more direct way of monitoring electrostatic interactions because of the sensitivity of the phosphorus NMR chemical shift to orbital perturbations caused by charged groups and also to any related changes in the bond angles (or hybridization) around the phosphorus atoms (Blackburn et al., 1971; Gorenstein & Kar, 1975). In this paper we report the use of a phosphonium analogue of phosphocholine that enables the electrostatic interactions at both ends of the hapten to be monitored by ^{31}P NMR.

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[1] Abbreviations: NMR, nuclear magnetic resonance; IgA, immunoglobin A; Fab′ fragment, N-terminal half of heavy chain and light chain; V_L, variable domain of a light chain; V_H, variable domain of a heavy chain.

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^{31}P NMR studies have been used previously to probe the ionization state of the hapten phosphate group (Gettins et al., 1977; Goetze & Richards, 1977a,b, 1978) when bound to M603 and related phosphocholine-binding proteins. In this way it has been shown that the binding energies of the mono- and dianionic forms are very similar. This has also been confirmed (Goetze & Richards, 1978) from a detailed comparison, as a function of pH, of the binding of esters of phosphocholine such as L-α-glycerophosphocholine (which can only exist in the monoanionic form above ca. pH 2) with phosphocholine. The relative affinities of these two haptens approximately correlated with the degree of ionization of phosphocholine as monitored by ^{31}P NMR.

In addition to ^{31}P NMR being used to probe the role of ionizable groups, ^1H NMR studies can also give important molecular details about the environment of the hapten. For instance the generation of high-resolution proton NMR difference spectra (Dwek et al., 1975) on addition of hapten can help in providing a measure of the degree of conformational changes in the protein on hapten binding. These spectra highlight those residues whose environment is changed on addition of hapten. Our preliminary results for M603 (Gettins et al., 1977) suggested that there were no large conformational changes on addition of phosphocholine. However, the difference specta were consistent with the binding site containing several aromatic residues. Here we also use ^1H NMR on choline to probe this subsite further with respect to the position of the aromatic rings relative to the hapten by analyzing the change in chemical shifts of the hapten resonances on binding to the antibody (Perkins & Dwek, 1980). From the refined crystal structure we know the number and type of aromatic residues in the site and their disposition. This provides a starting point for the NMR calculations for which the resolution of the pairwise interactions may often be better than ~0.05 nm (Perkins & Dwek, 1980).

Materials and Methods

Materials. $Me_3P^+CH_2CH_2OPO_3H^-$ was a gift from Dr. Hands (Oxford), and phosphocholine and choline were obtained from from Sigma Chemical Co., St. Louis, MO.

Fab' Fragment. The Fab' fragment of M603 was prepared from IgA by digestion with pepsin (Inbar et al., 1971). After purification by gel filtration the fragment was dialyzed against ammonium bicarbonate solution at pH 7.5 and freeze-dried.

Antibody Solutions. Samples were prepared by dissolving freeze-dried material in 99.8% ^2H_2O containing 150 mM NaCl and 0.02% sodium azide. The pH was adjusted with ^2HCl and NaO^2H and was not corrected for deuterium isotope effects. Concentrations were determined by measurement of absorbance at 280 nm with $E^{1\%}$ = 14.0 (Inbar et al., 1971).

NMR Methods. (*i*) *^1H NMR.* Spectra were recorded at 270 MHz on a Bruker spectrometer, as previously described (Hoult & Richards, 1975), operating in the Fourier-transform mode with quadrature phase sensitive detection (Hoult, 1978). A 70° pulse (12–15 μs) was employed with a repeat of 0.6 s and a sweep width of 400 Hz. Residual intensity from solvent water protons was removed by a selective saturating pulse of 0.4-s duration, applied before the nonselective 70° pulse, with a delay of 800 μs between the two pulses. An internal deuterium field frequency lock was used. The samples (0.35 cm^3) were contained in 5 mm o.d. sample tubes. Chemical shifts are quoted as parts per million of the applied field, downfield from sodium 4,4-dimethyl-4-silapentanesulfonate as external standard.

(*ii*) *^{31}P NMR.* Spectra were recorded at 36.43 MHz on a Bruker WH90 for which spherical sample holders of 0.4-cm^3 volume inserted into 10-mm tubes were used. Proton decoupling was employed, and spectra were recorded in the Fourier-transform mode with a pulse angle of 60°. Chemical shifts are quoted relative to 85% v/v phosphoric acid as external standard.

Ring Current Calculations. The hapten and surrounding aromatic residues were constructed on a scale of 2 cm = 0.1 nm with standard Kendrew skeletal components. Ring current shifts were calculated by the method of Johnson & Bovey (1958) with the scaling factors of Perkins & Dwek (1980) by use of a FORTRAN computer program. Unrestricted methyl group rotation about the N–C bond was allowed as suggested from the ^{13}C line-width measurements of Goetze & Richards (1977a).

Table I: Chemical Shifts and Their pH Dependence ($\Delta\delta$) for $Me_3N^+CH_2CH_2OPO_3H^-$ and $Me_3P^+CH_2CH_2OPO_3H^-$ [a]

	^1H shifts						
	Me	$\Delta\delta$	α-CH_2	$\Delta\delta$	β-CH_2	$\Delta\delta$	pK_a
Me_3P^+	1.91	0	2.58	−0.10	4.21	−0.15	6.30
Me_3N^+	3.21	−0.01	3.59	−0.07	4.16	−0.13	5.45

	^{31}P shifts				
	PO_4	$\Delta\delta$	P^+	$\Delta\delta$	pK_a
Me_3P^+	−3.65	+3.76	−26.16	0	6.29
Me_3N^+	−3.40	+3.84			5.55

[a] Shifts are given for the dianionic species.

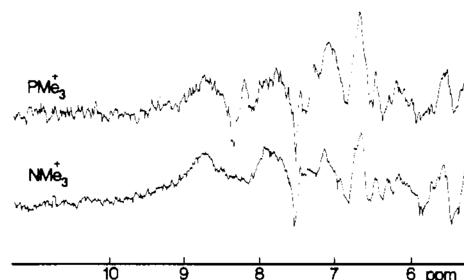

FIGURE 1: 270-MHz ^1H NMR difference spectra for $Me_3P^+CH_2CH_2OPO_3^{2-}$ (upper) and $Me_3N^+CH_2CH_2OPO_3^{2-}$ (lower) binding to M603 at pH* 7.45.

Results

Phosphonium Analogue of Phosphocholine ($Me_3P^+CH_2CH_2OPO_3^{2-}$). This has a ^{31}P NMR spectrum consisting of two resonances separated by over 20 ppm, with the lower field one due to the quaternary phosphorus. In Table I, the proton, as well as phosphorus, chemical shifts of this compound are compared with those of phosphocholine. The effects of protonation of the phosphate dianion are also given. Despite the differences in chemical shift arising from phosphorus substitution for nitrogen, the changes caused by protonation of the phosphate ester dianion are nearly identical with those of phosphocholine for all four resonances. This suggests that the phosphorus analogue should be a good model for the normal phosphocholine. This is confirmed for the antibody used here from the ^1H difference spectra for the two haptens, obtained after binding to M603. Figure 1 shows that the same few aromatic resonances are perturbed in each case, indicating that the two haptens bind in an almost identical manner in the combining site.

An important advantage of using the analogue is that the phosphonium resonance does not sense changes in the ionization state of the phosphate group (Table I) at the other end of the molecule. This means that the changes in chemical shift

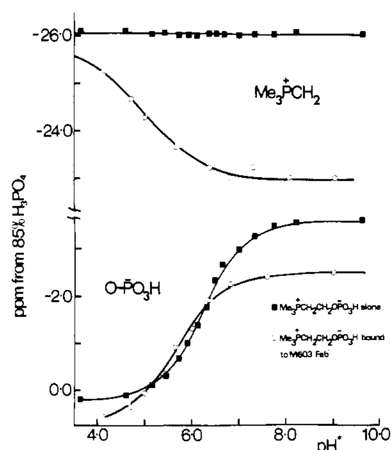

FIGURE 2: pH titration behavior of the two phosphorus nuclei in $Me_3P^+CH_2CH_2OPO_3^{2-}$, followed by ^{31}P NMR at 36.43 MHz.

of the phosphonium resonance upon binding to antibody can be attributed to local effects from the choline subsite of the protein.

^{31}P NMR Titrations. The pH dependence of the chemical shift of both phosphorus nuclei of the hapten in the presence of an excess of antibody and in its absence is given in Figure 2. As a consequence of binding to M603, the phosphate resonance is shifted upfield at alkaline pH (though by 1.1 ppm compared with a shift change of 1.8 ppm with phosphocholine). The pK_a is lowered from 6.3 to 5.8. This decrease in pK_a by 0.5 unit is the same as for normal phosphocholine (Gettins et al., 1977; Goetze & Richards, 1977a,b, 1978), suggesting that similar molecular interactions occur in both cases. For the phosphonium phosphorus signal, very different behavior is observed. At high pH there is an upfield shift of 3 ppm upon binding. The chemical shift then titrates to lower field as the pH is reduced, reaching a value only 0.6 ppm upfield of the free hapten resonance frequency at pH 4.0. Below this pH the sample precipitates. The line width of the phosphonium phosphorus signal remains constant with pH, indicating that conditions of slow exchange are valid (Dwek 1973) and that the shifts really represent those of the bound hapten at all pH values. This was also true for the phosphate resonances except possibly at the lowest pH value studied when a very slight broadening was detectable.

^1H NMR Hapten Shifts. Phosphocholine binds to M603 with a K_d of 5 μM, at pH 7.4. The value of the exchange rate k_{off} is $\sim$10 s^{-1} (Goetze & Richards, 1977a) and is such that slow exchange conditions obtain for hapten and protein proton resonances. The previous finding (Gettins et al., 1977) that the hapten resonances were in fast exchange is erroneous and possibly arose from slight mismatching of difference spectra. By contrast, although choline has a considerably lower affinity of only 1.2 mM (Goetze & Richards, 1977a) because of the absence of the phosphate head group, all its resonances are in fast exchange at 303 kHz and 270 MHz. Presumably this reflects the greater value of k_{off} for choline. This can be estimated from the relationship $K_d = k_{off}/k_{on}$, by assuming that k_{on} is approximately the same as that for phosphocholine, as $k_{off} \sim 2.5 \times 10^3$ s^{-1} so that resonances whose shifts are $\sim$1.4 ppm between free and bound states should be in fast exchange (Dwek, 1973). This hapten was therefore used in an attempt to measure the perturbations on the hapten chemical shifts from the aromatic rings in the binding site.

The dependence of chemical shift on the fraction bound is shown in Figure 3 for the methyl resonances, which, being much more intense than those of the methylenes, can be more

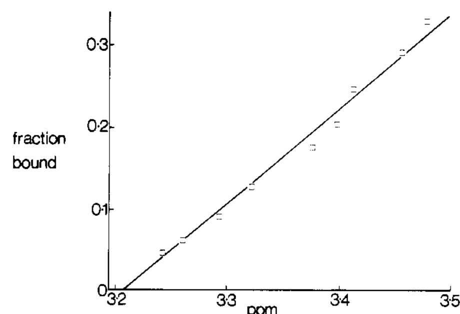

FIGURE 3: Plot of chemical shift of methyl resonance of choline as a function of fraction bound to M603. The slope gives the full change in chemical shift on binding.

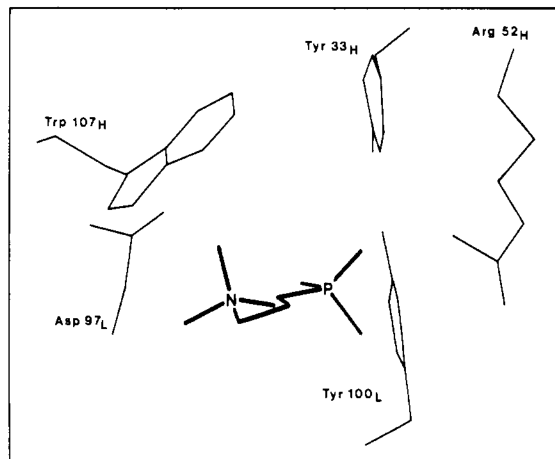

FIGURE 4: Hapten contact residues in M603 kindly supplied by Dr. D. R. Davies based on the coordinates from refinement at 2.7 Å together with a 3.1-Å difference Fourier map from phosphocholine. The numbering system is that of Davies and different somewhat from the immunologists numbering system.

Table II: Changes in Chemical Shift of Three Proton Resonances of Choline Chloride on Binding to M603 Fab′ [a]

	$^+NMe_3$	α-CH_2	β-CH_2
shift ratio	1	0.73	0.38
change in chemical shift (ppm)	+0.88	+0.64 [b]	+0.34 [b]

[a] A plus sign represents an upfield shift. [b] These values were determined by multiplying the value of +0.88 ppm for the methyl resonance by the observed shift ratio.

easily seen at lower hapten concentrations. The fraction bound was calculated from the the published dissociation constant. From the slope, the fully bound chemical shift change was calculated. The shift changes for the α- and β-methylene protons were derived from the value for the methyl resonance by using the ratio of the chemical shift changes between them as scaling factors. Table II gives the experimental shift changes, which are all large and upfield.

Ring Current Calculations. For the purpose of ring current calculations, it is necessary to assume that all, or a major portion, of the large upfield shifts of the choline resonances upon binding are attributable to the nearby aromatic side chains. The crystal structure (Figure 4) places tryptophan-107_H, tyrosine-100_L, and tyrosine-33_H in a position to interact with the hapten. However, only tryptophan-107_H and tyrosine-100_L are close enough to the hapten protons to significantly contribute to the ring current shifts. The side chain conformations of these that give best agreement with the experimental results are shown in Figure 5 (Table III) and are

Table III: Ring Current Shifts (ppm) Calculated from Tryptophan-107_H and Tyrosine-100_L on Choline as Shown in Figure 5[a]

	α-CH_2	β-CH_2	$^+NMe_3$
tyrosine-100_L	0.27	0.16	0.13
tryptophan-107_H	0.27	0.25	0.63
total	0.54	0.41	0.76

[a] The values are based on the tables of Johnson & Bovey (1958) with scaling factors for tryptophan as in Perkins & Dwek (1980).

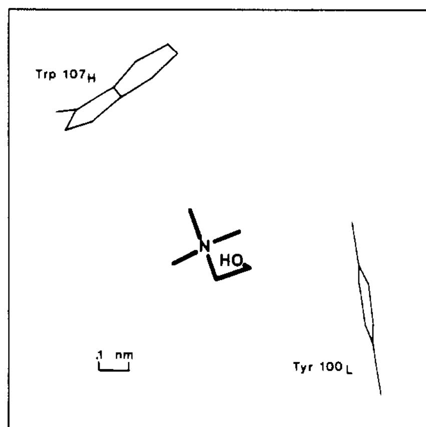

FIGURE 5: Conformations of Trp-107_H and Tyr-100_L relative to hapten choline as used for NMR ring current calculations.

in very similar orientations to those expected on the basis of the crystal structure.

Discussion

The ^{31}P NMR studies reported here have probed the two charged subsites, which are essential for binding the phosphocholine. With the crystal structure as a basis, the ^1H NMR studies have shown the position of the aromatic hapten contact residue that can account for the chemical shifts on the hapten protons.

From the similarity of the proton aromatic region difference spectra for phosphocholine and its phosphonium analogue binding to M603, and the comparable effects in the ^{31}P NMR spectra, it can be concluded that the analogue is indeed a good model. As with phosphocholine the lowering of the pK_a value of the phosphate group by 0.5 unit on binding to M603 is in agreement with previous results (Gettins et al., 1977; Goetze & Richards, 1977a,b, 1978) and again confirms that the binding energies of the mono- and dianionic forms are very similar. A decrease in pK_a is also expected if the phosphate group is hydrogen bonded to Tyr-33_H and Arg-52_H as suggested from X-ray data. However, the magnitude of the decrease is difficult to interpret since there could be an opposing effect, tending to increase the pK_a value (Dower & Dwek, 1979), resulting from any desolvation of the phosphate group on being transferred to the more hydrophobic environment of the combining site.

At the other end of the analogue, observation of the quaternary phosphorus that carries the normal positive charge is most likely to reveal electrostatic interactions between hapten and protein. The phosphonium phosphorus cannot change its ionization state and, in the uncomplexed state, has a ^{31}P chemical shift totally dependent on the phosphate's charge state. It therefore appears that its titration behavior as a function of pH in the bound form is a reflection of a group (or groups) on the protein, which ionize(s) with a $pK_a \leq 5.0$. Inspection of the refined binding site determined from the X-ray crystallographic data (Figure 4) suggests that the phosphonium ion is sensing the ionization of Asp-97_L. This ionization could also be the origin of the small (<0.5 ppm) ^{31}P chemical shifts of the phosphate group between pH 4 and 6 observed with L-α-glycerophosphocholine (Goetze & Richards, 1978). This hapten exists only as the monoanion above pH 2. Presumably this shift is masked in cases where the phosphate group itself begins to ionize over this range.

As with the upfield shift of the phosphate ^{31}P resonance on binding phosphocholine (Gettins et al., 1977) there is difficulty in accounting for the upfield shift of 3 ppm at high pH on the phosphonium ^{31}P resonance. The data in Figure 2 show that this resonance titrates back to that of the free hapten, at low pH. The 3 ppm chemical shift change may be due to the presence of the negatively charged form of the side-chain residue Asp-97_L, which may be in contact with the choline moiety. However, with the uncertainty in the explanation of phosphorus shifts the precise mechanism remains unclear.

For phosphocholine-binding antibodies, the use of ^{31}P NMR to monitor the phosphate interaction (Gettins et al., 1977; Goetze & Richards, 1977a,b, 1978) or the use of the phosphonium analogue to monitor the negatively charged groups forming the choline subsite is a powerful way of looking at the two charged subsites. These subsites appear from binding studies (Leon & Young, 1971) to be essential in phosphocholine-binding antibodies. Since the extact position and properties, as well as the mere existence of these subsites, will affect the affinity, it is desirable to have the ^{31}P NMR data in addition to the binding constants (Goetze & Richards, 1978). Whereas changes in pK_a for the phosphate group and reflected ionizations in the phosphonium ion can be readily interpreted, the changes in chemical shift, as with proton shifts, are open to wide interpretation. Nevertheless, a change can still be used qualitatively for comparative studies for related antibodies, as has been successfully demonstrated by Goetze & Richards (1977b, 1978).

Finally, we note that the ^1H NMR shifts on choline can almost be completely accounted for on the basis of ring current interactions from Trp-107_H and Tyr-100_L. This together with the other results in this paper and those reported previously (Gettins et al., 1977; Goetze & Richards, 1977a) is consistent with the major interactions between hapten and antibody suggested on the basis of the refined crystal structure (Figure 4).

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Activation of Poly(adenosine diphosphate ribose) Polymerase by SV 40 Minichromosomes: Effects of Deoxyribonucleic Acid Damage and Histone H1†

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ABSTRACT: Poly(ADP-ribose) polymerase is a chromosomal enzyme that is completely dependent on added DNA for activity. The ability of DNA molecules to activate the polymerase appears to be enhanced by the presence of DNA damage. In the present study, we used SV 40 DNA and SV 40 minichromosomes to determine whether different types of DNA damage and different chromosomal components affect stimulation of polymerase activity. Treatment of SV 40 minichromosomes with agents or conditions that induced single-strand breaks increased their ability to stimulate poly(ADP-ribose) synthesis. This stimulation was enhanced by addition of histone H1 at a ratio of 1 μg of histone H1 to 1 μg of DNA. Higher ratios of histone H1 to DNA suppressed the ability of SV 40 minichromosomes containing single-strand breaks to stimulate enzyme activity. Treatment of SV 40 minichromosomes or SV 40 DNA with *Hae*III restriction endonuclease to produce double-strand breaks markedly stimulated poly(ADP-ribose) polymerase activity. The stimulation of poly(ADP-ribose) polymerase by double-strand breaks occurred in the absence of histone H1 and was further enhanced by adding histone H1 up to ratios of 2 to 1 relative to DNA. At higher ratios of histone H1 to DNA, the presence of the histone continued to enhance the poly(ADP-ribose) synthesis stimulated by double-strand breaks.

Poly(adenosine diphosphate ribose) polymerase is a chromosomal enzyme that catalyzes the polymerization of ADP-ribose moieties from NAD^+ (Hayaishi & Ueda, 1977; Purnell et al., 1980). In some cases, the polymers of ADP-ribose are covalently linked to chromosomal proteins (Hayaishi & Ueda, 1977; Purnell et al., 1980). Many studies now indicate that this enzyme has a role in the recognition and repair of DNA damage. For example, studies with nuclei isolated from HeLa cells and *Physarum polycephalum* show that DNA damage induced by alkylating agents results in increased poly(ADP-ribose) polymerase activity (Smulson et al., 1975; Whish et al., 1975; Sudhakar et al., 1979). Permeabilized lymphocytes with DNA damage induced by UV irradiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, *N*-acetoxy-2-(acetylamino)fluorene, and bleomycin have increased poly(ADP-ribose) polymerase activity (Berger et al., 1979), 3T3 cells depleted of NAD^+, the substrate for poly(ADP-ribose) polymerase, are unable to undergo *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced unscheduled DNA synthesis (Jacobson et al., 1980),

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