Monoclonal Antibodies Specific for Poly(dG)·Poly(dC) and Poly(dG)·Poly(dm^{5}C)†

Jeremy S. Lee,* Mary L. Woodsworth, and Laura J. P. Latimer

ABSTRACT: Most duplex DNAs that are in the "B" conformation are not immunogenic. One important exception is poly(dG)·poly(dC), which produces a good immune response even though, by many criteria, it adopts a conventional right-handed helix. In order to investigate what features are being recognized, monoclonal antibodies were prepared against poly(dG)·poly(dC) and the related polymer poly(dG)·poly(dm^{5}C). Jel 72, which is an immunoglobulin G, binds only to poly(dG)·poly(dC), while Jel 68, which is an immunoglobulin M, binds approximately 10-fold more strongly to poly(dG)·poly(dm^{5}C) than to poly(dG)·poly(dC). For both antibodies, no significant interaction could be detected with any other synthetic DNA duplexes including poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)] in both the "B" and "Z" forms, poly[d(Tm^{5}Cm^{5}C)]·poly[d(GGA)], and poly[d(TCC)]·poly[d(GGA)], poly(dI)·poly(dC), or poly(dI)·poly(dm^{5}C). The binding to poly(dG)·poly(dC) was inhibited by ethidium and by disruption of the DNA duplex, confirming that the antibodies were not recognizing single-stranded or multistranded structures. Furthermore, Jel 68 binds significantly to phage XP-12 DNA, which contains only m^{5}C residues and will precipitate this DNA in the absence of a second antibody. The results suggest that $(dG)_n$·$(dm^5C)_n$ sequences in natural DNA exist in recognizably distinct conformations.

It is abundantly clear that the vast majority of duplex DNA exists in the "B" conformation under physiological conditions. However, many structural variations have been documented (Leslie et al., 1980; Dickerson et al., 1982; Cantor, 1981, Dickerson, 1983), the most dramatic of which is "Z" DNA (Wang et al., 1979). The possibility that these different conformations have a significant function in living organisms is intriguing, especially since Z DNA has been found in a variety of eucaryotic chromosomes (Nordheim et al., 1981; Morgenegg et al., 1983; Zarling et al., 1984; Lipps et al., 1983) and supercoiled phage DNAs (Nordheim et al., 1982; Klysik et al., 1982; Haniford & Pulleyblank, 1983; Nordheim & Rich, 1983). Another candidate for an unusual sequence having possible functional significance is poly(dG)·poly(dC). Long stretches of this DNA have been found in bacteria (Szybalski et al., 1966) and shorter lengths are relatively common (Wells et al., 1977). Several lines of evidence suggest that poly(dG)·poly(dC) has a rather different structure from the classic B conformation.

First, the circular dichroism spectra are very unusual with the major positive band at 255 nm rather than at 280 nm found for B DNA and most other synthetic DNA duplexes (Gray et al., 1978; Marck & Thiele, 1978.) Second, X-ray fiber diffraction studies suggest that this DNA has a greater preference for the A conformation than does calf thymus DNA (Arnott & Selsing, 1974). Third, poly(dG)·poly(dC) is immunogenic (Stollar, 1970). All other unmodified DNA duplexes except possibly poly[d(GC)]·poly[d(GC)] and poly[d(TG)]·poly[d(CA)] produce only weak immune responses in animals, if at all (Lafer et al., 1982; Stollar, 1980; unpublished observations). On the other hand, the number of base pairs per turn is 10.6, which is indistinguishable from that of B DNA (Peck & Wang, 1981.); also, a short fragment of oligo(dG)·oligo(dC) adopted a B-type conformation in solution even when attached contiguously to an RNA–DNA duplex in the A conformation (Selsing et al., 1978)

Monoclonal antibodies specific for Z DNA are now being used extensively to probe the physical and functional properties of this unusual DNA. We anticipate that the monoclonal antibodies to poly(dG)·poly(dC) and poly(dG)·poly(dm^{5}C) described in this study can be used in the same way. In eucaryotes, although the major site of methylation is at CpG sequences, significant amounts of methylated CpC are also found (Grippo et al., 1968; Haigh et al., 1982; van der Ploeg & Flavell, 1980). In view of the dramatic effect that methylation has on the ability of poly[d(GC)]·poly[d(GC)] to undergo the B to Z transition (Behe & Felsenfeld, 1981), it seems possible that methylation of CpC sequences might also lead to structural changes.

Materials and Methods

Nucleic Acids. Poly(dG), poly(dI), poly(dC), poly(rG), poly(rI), poly(rC), poly(dG)·poly(dC), and poly[d(GC)]·poly[d(GC)] were purchased from P-L Biochemicals. Bacteriophage XP-12 DNA (Ehrlich et al., 1975) was a gift of Dr. M. Ehrlich, University of Tulane. Poly(dG)·poly(dm^{5}C) and poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)] and the other synthetic polymers containing 5-methylcytosine were synthesized with *Escherichia coli* DNA polymerase I and dm^{5}CTP (P-L Biochemicals) as described previously (Morgan et al., 1974; Gill et al., 1974; Evans et al., 1982). Poly(dm^{5}C) was prepared by depurination of the duplex (Harwood & Wells, 1970). Concentrations were calculated from the absorbance measurements with extinction coefficients listed previously (Morgan et al., 1979; Wells et al., 1970) except for poly(dG)·poly(dm^{5}C) and XP-12 DNA, where E_{260} was assumed to be 6600 M^{-1}. Several preparations of poly(dG)·poly(dC) containing an excess of poly(dG) were also prepared by the method of Litman (1971).

Poly(dI)·poly(rC), poly(dI)·poly(dm^{5}C), poly(dI)·poly(dC), poly(rG)·poly(dC), poly(rI)·poly(dm^{5}C), and poly(rI)·poly(dC) were formed from the appropriate single-stranded nucleic acids by annealing equimolar amounts in 0.1 M NaCl. Duplex formation was monitored by the ethidium bromide fluorescence assay (see below).

Monoclonal Antibodies. Two female C57 black mice were given three injections at 10-day intervals of 25 μg of either poly(dG)·poly(dC) or poly(dG)·poly(dm^{5}C) coupled to 25 μg of methylated bovine serum albumin. The first two injections were given intraperitoneally after emulsification with Freund's adjuvant while the final injection was intravenous. Three days

† From the Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. *Received October 27, 1983.* Supported by the Medical Research Council of Canada and the Saskatchewan Health Research Board.

0006-2960/84/0423-3277$01.50/0

later, the spleens were removed, and the splenocytes were cultured for 24 h in 20 μg/mL lipopolysaccharide (Sigma). The fusion protocol with myeloma MOPC 315.43 and culture conditions have been described previously except that all media contained 0.1 μM sodium selenite (Lee et al., 1981).

Ten days later, testing was initiated with a solid-phase radioimmunoassay (SPRIA) (see below). For the fusion with poly(dG)·poly(dC) as antigen, only one positive cell line (Jel 72) was successfully cloned. When the methylated polymer was used as antigen, 15 cell lines were cloned but only one of these (Jel 68) continued to secrete antibodies having specificity for poly(dG)·poly(dm^{5}C). Ascites fluid was prepared for these two cell lines and was purified on a Sephacryl S-200 column as described previously (Lee et al., 1982). The column profile and titers of the various fractions demonstrated that Jel 72 was an immunoglobulin G (IgG) and Jel 68 an immunoglobulin M (IgM).

Solid-Phase Radioimmunoassays. For screening of the fusions, the assays were performed as described earlier with DNA bound to poly(vinyl chloride) (PVC) plates as the solid phase; the goat anti-mouse I^{125}-labeled second antibody was purchased from Amersham (Lee et al., 1981). For testing the specificity of the hybridomas, PVC plates were coated with 2 μg/mL of the appropriate nucleic acid in PBS buffer (2.7 mM KCl, 137 mM NaCl, 10 mM Na_2HPO_4, 1.4 mM KH_2PO_4, final pH of 7.2), and a 5-fold dilution of the cell culture supernatant was used routinely. For competition experiments, the competitor in PBS was added first, followed by the DNA-binding antibody, and then the assay was continued as usual.

Other Techniques. Thermal denaturation profiles were measured at 260 nm on a Gilford 260 spectrophotometer equipped with a thermal programmer. Samples of 300 μL were heated at a rate of 0.5 °C/min in a buffer of 2.5 mM tris(hydroxymethyl)aminomethane (Tris-HCl), pH 8.0, and 25 μM ethylenediaminetetraacetic acid (EDTA). Ethidium bromide fluorescence measurements have been described previously (Morgan et al., 1979). Direct precipitation of DNA (4.5 μg/mL) with antibodies was performed in 1.5-mL Eppendorf tubes in PBS buffer. After a 30-min incubation at 20 °C, the tubes were centrifuged at 27000*g* for 30 min before measuring the DNA concentration in the supernatant with the ethidium bromide fluorescence assay.

Results

Characterization of Poly(dG)·Poly(dm^{5}C). The reaction conditions used for the synthesis of the methylated homopolymer were identical with those for poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)] except that different DNAs were used to prime the reaction. Thus, we were concerned that our putative preparation of poly(dG)·poly(dm^{5}C) was not contaminated with the alternating polymer. Figure 1 shows thermal denaturation profiles for poly(dG)·poly(dC), poly(dG)·poly(dm^{5}C), and poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)]. As expected, poly(dG)·poly(dm^{5}C) melts at a considerably higher temperature (86 °C) than the unmethylated polymer (71 °C) but approximately 3 °C lower than the methylated alternating polymer (89 °C). The small increase in hyperchromicity observed for poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)] before the main transition is due to the presence of poly[d(GC)]·poly[d(GC)] used to prime the synthesis. Similar contamination of poly(dG)·poly(dm^{5}C) with poly(dG)·poly(dC) was not observed since in this case replication was several hundredfold. Because of the sharpness of the transitions, preparations of poly(dG)·poly(dm^{5}C) are estimated to be less than 5% contaminated with the alternating polymer.

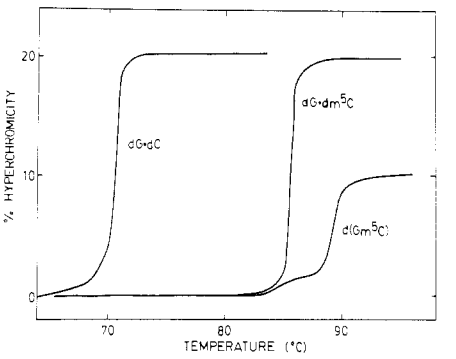

FIGURE 1: Thermal denaturation profiles for poly(dG)·poly(dC), poly(dG)·poly(dm^{5}C), and poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)]. See text for details.

Table I: Solid-Phase Radioimmunoassay Results for Jel 68 and Jel 72 Hybridoma Supernatants Binding to Various Nucleic Acids[a]

polymer	Jel 68	Jel 72
poly(dG)·poly(dm^{5}C)	100	<5
poly(dG)·poly(dC)	70	100
poly[d(GC)]·poly[d(GC)]	<5	<5
poly[d(Gm^{5}C)]·poly[d(Gm^{5}C)][b]	<5	<5
poly(dG)	<5	<5
poly(dC)	<5	<5
poly(dm^{5}C)	<5	<5
poly[d(TCC)]·poly[d(GGA)]	<5	<5
poly[d(Tm^{5}Cm^{5}C)]·poly[d(GGA)]	<5	<5
poly(dI)·poly(rC)	<5	<5
poly(dI)·poly(dm^{5}C)	<5	<5
poly(dI)·poly(dC)	<5	<5
poly(rG)·poly(dC)	27	<5
poly(rI)·poly(dm^{5}C)	6	<5
poly(rI)·poly(dC)	<5	<5

[a] Results are expressed as a percentage of maximum binding after subtraction of the background. For Jel 68 and Jel 72, the maximum cpm were approximately 2000 and 6000, respectively, with a background cpm of 200 in both cases. [b] In both the B and Z forms. The Z form was generated in 5 mM Mg^{2+}. This level of Mg^{2+} had no effect on the binding to poly(dG)·poly(dC).

Another potential problem with the synthesis of polymers containing poly(dG) is the propensity of *E. coli* polymerase I to replicate more poly(dG) than poly(dC) (Radding et al., 1962), giving rise to triplexes and other complex structures (Marck & Thiele, 1978; Wells et al., 1970.) A relatively simple method for assessing the strand ratios of polymers is to measure the ethidium bromide fluorescence to absorbance ratio (relative fluorescence). Since ethidium only shows enhanced fluorescence with duplexes, a low relative fluorescence would indicate an unequal strand ratio (Morgan et al., 1979). The relative fluorescence for various preparations of poly(dG)·poly(dC) was from 60 to 70, while that for poly(dG)·poly(dm^{5}C) was found to be 71. This is to be compared with a relative fluorescence of 70 for calf thymus DNA and values of 65–75 for most synthetic duplex DNAs (unpublished observations; Morgan et al., 1979). Therefore, we estimate that the strand ratio of poly(dG)·poly(dm^{5}C) does not differ from unity by more than 20%.

Specificity of Monoclonal Antibodies. The specificity patterns of the monoclonal antibodies were investigated initially by SPRIA as shown in Table I. Jel 72 binds only to poly(dG)·poly(dC) while Jel 68 binds preferentially to poly(dG)·poly(dm^{5}C). For the latter, there was also a very weak interaction with poly(rI)·poly(dm^{5}C) and poly(rG)·poly(dC). For both antibodies, there was no detectable interaction with poly[d(TCC)]·poly[d(GGA)] or poly[d(Tm^{5}Cm^{5}C)]·poly[d-

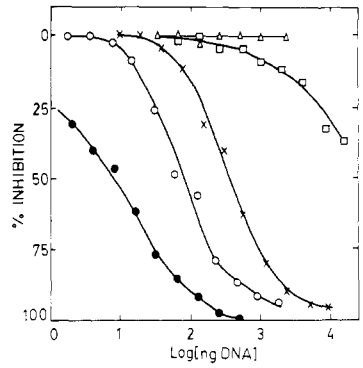

FIGURE 2: Competition binding experiments with hybridoma supernatants from Jel 68 and Jel 72. The percent inhibition of binding to poly(dG)·poly(dC) is shown as a function of the amount of DNA added as competitor in the SPRIA. For Jel 72: (×) poly(dG)·poly(dC); (△) poly(dG)·poly(dm^{5}C). For Jel 68: (○) poly(dG)·poly(dC); (●) poly(dG)·poly(dm^{5}C); (□) DNA from phage XP-12.

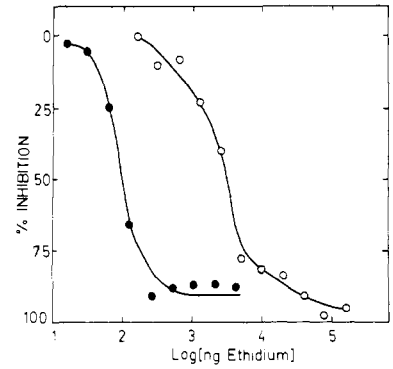

FIGURE 3: Competition binding experiments with hybridoma supernatants. The percent inhibition of binding to poly(dG)·poly(dC) is shown as a function of ethidium added as competitor in the SPRIA: (●) Jel 68; (○) Jel 72.

(GGA)], which is of interest because these duplexes might be considered to be most closely related in sequence to poly(dG)·poly(dC) and the methylated polymer. Because of the subtlety of DNA structure, we also tested the antibodies against: poly[d(AT)]·poly[d(AT)], poly(dA)·poly(dT), poly[d(ATC)]·poly[d(GAT)] (+ methylated analogue), poly[d(TTG)]·poly[d(CAA)] (+ methylated analogue), poly[d(TTC)]·poly[d(GAA)] (+ methylated analogue), poly[d(TG)]·poly[d(CA)] (+ methylated analogue) poly[d(TC)]·poly [d(GA)] (+ methylated analogue), as well as native and heat-denatured calf thymus DNA. In no case was there any detectable interaction. (The synthesis and properties of these methylated polymers will be described elsewhere.)

Although SPRIA is useful as an initial screen for positive antigens, more detailed binding studies were performed with competition experiments (Figure 2). For Jel 68, poly(dG)·poly(dm^{5}C) is approximately 10-fold more effective as a competitor than the unmethylated polymer, confirming the indications of the SPRIA. No competition could be seen with poly[d(TCC)]·poly[d(GGA)] at the highest concentrations available, but intriguingly, at very high concentrations XP-12 DNA and calf thymus DNA showed some binding. For XP-12 DNA, which contains only m^{5}C residues (Ehrlich et al., 1975), this level of inhibition is 0.05% of that shown by poly(dG)·poly(dm^{5}C), which corresponds to 25 base pairs in the phage genome. The inhibition shown by calf thymus DNA was barely significant (data not shown) so that recognizable binding sites on this DNA must be exceedingly rare. For Jel 72, higher concentrations of poly(dG)·poly(dC) were required to compete effectively, and no competition was observed with poly(dG)·poly(dm^{5}C), poly[d(TCC)]·poly[d(GGA)], or calf thymus DNA. We conclude that the binding constant of Jel 72 to poly(dG)·poly(dC) is at least 100-fold greater than to any other nucleic acid.

Poly(dG)·poly(dC) [and therefore, presumably, poly(dG)·poly(dm^{5}C)] is notorious for forming complex structures involving triplexes (Marck & Thiele, 1978; Wells et al., 1970) and tetraplexes (Lee et al., 1980). Consequently, the possibility was considered that the antibodies were binding to a minor component of the synthetic DNAs. However, two lines of evidence suggest that the antigenic determinant for both antibodies is duplex DNA.

First, as shown in Figure 3, ethidium competes effectively with both antibodies for binding to poly(dG)·poly(dC). Since ethidium does not bind to G-C containing triplexes (Lee et al., 1979), tetraplex poly(dG) (Morgan et al., 1979; Lee et al., 1980), or single-stranded poly(dC), presumably the inhibition is occurring at the level of the duplex. Although higher levels of ethidium are required to inhibit the binding of Jel 72 compared to Jel 68, this may simply reflect a difference in binding constant or site size between the two antibodies. Second, deliberate attempts to destroy the duplex of poly(dG)·poly(dC) or to create complex structures lead to a reduction in binding as judged by competition experiments. This is shown in Table II. The "poly(dG)-rich" sample was synthesized by *E. coli* polymerase I (Litman, 1971) and, judging by the low relative fluorescence compared to the control, contains severalfold excess of poly(dG). The "pH 5 treated" sample was heated at 60 °C for 2 h at pH 5 to encourage formation of poly(dC)·poly(dG)·poly(dC$^+$) triplexes before being returned to neutral pH. This dismutation also leads to a lowering of the relative fluorescence (Lee et al., 1979). The "heat-denatured" sample was treated at 100 °C for 10 min before being quenched in ice, again leading to a drop in the relative fluorescence indicative of the presence of nonduplex structures. Lastly, the "poly(dG) + poly(dC)" sample was an equimolar mixture of the two single-stranded DNAs that had not been annealed to form the duplex, giving rise to a very low relative fluorescence. For both Jel 68 and Jel 72, these nonduplex DNAs competed less efficiently than the control duplex poly(dG)·poly(dC). In the case of Jel 72, there is a reasonable correlation between relative binding and relative fluorescence, but for Jel 68, the heat-denatured duplex competes significantly better than expected on the basis of its relative fluorescence. Again, these differences probably reflect the different antigenic determinants of the two antibodies. However, the important point is that none of these treatments leads to an improvement in the relative binding of the polymers, which would be expected if the antibodies were recognizing structures other than duplexes.

Experiments were also performed that demonstrated the binding of Jel 68 to XP-12 DNA by a direct method. Figure

Table II: Relative Binding of Jel 68 and Jel 72 to Complex Structures Related to Poly(dG)·Poly(dC)[a]

nucleic acid[b]	relative fluorescence	relative binding	
		Jel 68	Jel 72
control poly(dG)·poly(dC)	63	1	1
poly(dG)-rich duplex	32	0.3	0.7
pH 5 treated duplex	30	0.7	0.6
heat-denatured duplex	23	0.8	0.08
poly(dG) + poly(dC)	3	<0.05	<0.05

[a] The relative binding was estimated from competition experiments, as described in Figure 2. [b] See text for details on the preparation of these DNAs.

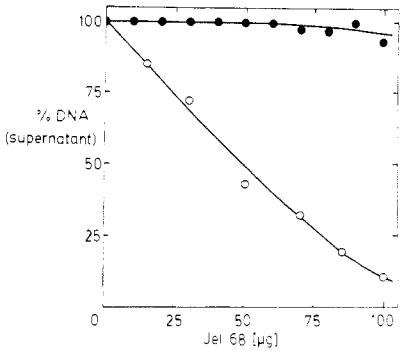

FIGURE 4: Direct precipitation of DNA by Jel 68, purified from ascites fluid. The percent DNA remaining in the supernatant is shown as a function of the antibody added: (○) DNA from phage XP-12; (●) calf thymus DNA.

4 shows the precipitation of XP-12 DNA in the absence of a second antibody (i.e., an anti-mouse antibody). Presumably, binding sites are scattered infrequently along the DNA so that the antibody cross-links different DNA molecules, forming a high molecular weight complex. Calf thymus DNA, on the other hand, is not precipitated to any significant extent; potential binding sites are expected to be less frequent with calf thymus DNA since the G + C content is only 42% compared to 67% G + m^5C content for XP-12 DNA (Ehrlich et al., 1975). Similar experiments with Jel 72 did not give rise to any significant precipitation of DNA, but the use of a second antibody is expected to obviate this problem.

Discussion

Previously, rabbit antibodies have been produced that showed considerable specificity for poly(dG)·poly(dC) although there was no clear demonstration that it was a duplex structure that was being recognized (Stollar, 1970). In the present study, this problem has been overcome by deliberately preparing nonduplex structures related to poly(dG)·poly(dC). Therefore, there can be little doubt that these monoclonal antibodies are binding duplex DNA (Table II).

The specificity of certain antibodies for Z DNA is not unexpected when the unique left-handed structure of this polymer is considered. The absolute specificity of Jel 72 for poly(dG)·poly(dC), therefore, is certainly surprising, considering that this homopolymer is generally thought to adopt a conventional B-type conformation (Selsing et al., 1978; Arnott & Selsing, 1974). It would be mere speculation to suggest what features of the helix Jel 72 is recognizing. Nor is it clear how to approach this problem since (apparently) small modifications of poly(dG)·poly(dC) such as conversion of G to I or C to m^5C lead to complete loss of binding. Jel 68, on the other hand, will tolerate the modification of C to m^5C, and indeed, poly(dG)·poly(dm^5C) is the preferred antigen. Preliminary circular dichroism studies (Dr. J. H. van de Sande, unpublished experiments) imply that the methylated polymer adopts a different conformation compared to poly(dG)·poly(dC) or B DNA. Therefore, the antibody may be recognizing structural differences between the methylated and unmethylated polymers, but a positive interaction with the methyl group of cytosine itself cannot be excluded.

Taken together, Jel 68 and 72 behave rather like a pair of restriction endonucleases (such as *Msp*I and *Hpa*II) (Bird & Southern, 1980), one of which will only cleave at the restriction site if the sequence is unmethylated. Indeed, if naturally occurring GC-rich sequences are long enough, then it may be possible to use the antibodies as probes for methylation of CpC residues outside the sequences normally amenable to restriction analysis. Moreover, Jel 72 itself may serve as an excellent model for gene regulation based upon a specific lack of protein binding when a sequence becomes methylated (Holliday & Pugh, 1975).

The demonstration that Jel 68 will bind to XP-12 DNA, albeit to a very limited extent, suggests that structures akin to poly(dG)·poly(dC) exist in vivo. Since antibodies can specifically bind these sequences, then perhaps regulatory proteins can do so as well. Future experiments will be directed toward assessing the potential role of these sequences in small genomes.

Registry No. Poly(dG)·poly(dm^5C), 90150-74-6; poly(dG)·poly(dC), 25512-84-9; poly[d(Gm^5C)], 51853-63-5.

References

Arnott, S., & Selsing, E. (1974) *J. Mol. Biol. 88*, 551–552.
Behe, M., & Felsenfeld, G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1619–1623.
Bird, A. P., & Southern, E. M. (1980) *J. Mol. Biol. 118*, 27–47.
Cantor, C. R. (1981) *Cell (Cambridge, Mass.) 25*, 293–295.
Dickerson, R. E. (1983) *J. Mol. Biol. 166*, 419–441.
Dickerson, R. E., Drew, H. R., Conner, B. N., Wing, R. M., Fratini, A. V., & Kohka, M. L. (1982) *Science (Washington, D.C.) 216*, 475–485.
Ehrlich, M., Ehrlich, H., & Mayo, J. A. (1975) *Biochim. Biophys. Acta 395*, 109–119.
Evans, D. H., Lee, J. S., Morgan, A. R., & Olsen, R. K. (1982) *Can. J. Biochem 60*, 131–136.
Gill, J. E., Mazrimas, J. A., & Bishop, C. C. (1974) *Biochim. Biophys. Acta 335*, 330–348.
Gray, D. M., Morgan, A. R., & Ratliff, R. L. (1978) *Nucleic Acids Res. 5*, 3679–3695.
Grippo, P., Iaccarino, M., Parisi, R., & Scarano, E. (1968) *J. Mol. Biol. 36*, 195–208.
Haigh, L. S., Owens, B. B., Hellewell, S., & Ingram, V. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5332–5336.
Haniford, D. B., & Pulleyblank, D. E. (1983) *Nature (London) 302*, 623–634.
Harwood, S. J., & Wells, R. D. (1970) *J. Biol. Chem. 245*, 5625–5634.
Holliday, R., & Pugh, J. E. (1975) *Science (Washington, D.C.) 187*, 226–232.
Klysic, J., Stirdivant, S. M., & Wells, R. D. (1982) *J. Biol. Chem. 257*, 10152–10158.
Lafer, E. M., Moller, A., Nordheim, A., Rich, A., & Stollar, B. D. (1982) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 41*, 1024.
Lee, J. S., Johnson, D. A., & Morgan, A. R. (1979) *Nucleic Acids Res. 6*, 3073–3092.
Lee, J. S., Evans, D. H., & Morgan, A. R. (1980) *Nucleic Acids Res. 8*, 4305–4320.
Lee, J. S., Lewis, J. R., Morgan, A. R., & Mosmann, T. R., & Singh, B. (1981) *Nucleic Acids Res. 9*, 1707–1721.
Lee, J. S., Dombroski, D. F., & Mosmann, T. R. (1982) *Biochemistry 21*, 4940–4950.
Leslie, A. G. W., Arnott, S., Chandrasekaran, R., & Ratliff, R. L. (1980) *J. Mol. Biol. 143*, 49–72.
Lipps, H. S., Nordheim, A., Lafer, E. M., Ammermann, D., Stollar, B. D., & Rich, A. (1983) *Cell (Cambridge, Mass.)* 435–441.
Litman, R. M. (1971) *J. Mol. Biol. 61*, 1–23.
Marck, C., & Thiele, D. (1978) *Nucleic Acids Res. 5*, 1017–1028.
Morgan, A. R., Coulter, M., Flintoff, W., & Paetkau, V. (1974) *Biochemistry 13*, 1596–1603.
Morgan, A. R., Lee, J. S., Pulleyblank, D. E., Murray, N. L., & Evans, D. H. (1979) *Nucleic Acids Res. 7*, 547–569.

Morgenegg, G., Celo, M. R., Malfoy, B., Leng, M., & Kuenzle, C. C. (1983) *Nature (London) 303*, 540–543.
Nordheim, A., & Rich, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 1821–1825.
Nordheim, A., Pardue, M. L., Lafer, E. M., Moller, A., Stollar, B. D., & Rich, A. (1981) *Nature (London) 294*, 417–422.
Nordheim, A., Lafer, E. M., Peck, L. J., Wang, J. C., Stollar, B. D., & Rich, A. (1982) *Cell (Cambridge, Mass.) 31*, 309–318.
Peck, J. L., & Wang, J. C. (1981) *Nature (London) 292*, 375–377.
Radding, C. M., Josse, J., & Kornberg, A. (1962) *J. Biol. Chem. 9*, 2869–2876.
Selsing, E., Wells, R. D., Early, T. A., & Kearns, D. R. (1978) *Nature (London) 275*, 249–250.
Stollar, B. D. (1970) *Science (Washington, D.C.) 169*, 609–611.
Stollar, B. D. (1980) *Methods Enzymol. 70*, 70–85.
Szybalski, W., Kubinski, H., & Sheldrick, P. (1966) *Cold Spring Harbor Symp. Quant. Biol. 31*, 123–127.
van der Ploeg, L. H. T., & Flavell, R. A. (1980) *Cell (Cambridge, Mass.) 19*, 947–958.
Wang, A. H. J., Quigley, G. J., Kolpak, F. J., Crawford, J. L., van Boom, J. H., van der Marel, G., & Rich, A. (1979) *Nature (London) 282*, 680–686.
Wells, R. D., Larson, J. E., Grant, R. C., Shortle, B. E., & Cantor, C. R. (1970) *J. Mol. Biol. 54*, 465–497.
Wells, R. D., Blakesley, R. W., Hardies, S. C., Horn, G. J., Larson, J. E., Selsing, E., Burd, J. F., Chan, H. W., Dodgson, J. B., Jensen, K. F., Nes, I. F., & Wartell, R. M. (1977) *CRC Crit. Rev. Biochem 4*, 305–340.
Zarling, D. A., Arndt-Jovin, D. J., Robert-Nicoud, M., McIntosh, L. P., Thomae, R., & Jovin, T. M. (1984) *J. Mol. Biol.* (in press).

6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-Monophosphate and 5′-Diphosphate: New Affinity Labels for Purine Nucleotide Sites in Proteins†

Roberta F. Colman,* Yu-Chu Huang, Marita M. King, and Markus Erb

ABSTRACT: Two new adenine nucleotide analogues have been synthesized and characterized: 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate and 5′-diphosphate. The bromoketo and dioxobutyl moieties have the ability to react with the nucleophilic side chains of several amino acids, as well as with arginine. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate reacts irreversibly with rabbit muscle pyruvate kinase, causing inactivation. Addition of ADP to the reaction mixture (in the presence of Mg^{2+}) markedly decreases the rate of inactivation. Pig heart NAD-dependent isocitrate dehydrogenase is allosterically activated by ADP, which reduces the K_m for isocitrate. 6-[(4-Bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-diphosphate reacts irreversibly with isocitrate dehydrogenase, causing, rapidly, a loss of the ability of ADP to increase the initial velocity of assays conducted at low isocitrate concentrations and, more slowly, inactivation. Addition of ADP to the reaction mixture (in the presence of Mn^{2+}) protects this enzyme against the loss of allosteric activation. It is proposed that the 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenine nucleotides react at the active site of pyruvate kinase and at the ADP activating site of isocitrate dehydrogenase and that these compounds may have general applicability as affinity labels of catalytic and regulatory adenine nucleotide sites in proteins.

Purine nucleotide analogues that have reactive functional groups at particular positions of the purine or ribose ring have proved effective in the affinity labeling of a variety of proteins (Colman, 1983). Desirable features of a purine nucleotide affinity label include structural similarity to the normal nucleotides (i.e., the presence of purine, ribose, and phosphate moieties), water solubility, reasonable stability in the pH range generally optimal for reaction with enzymes, and relatively high reactivity with many different types of amino acids since the participating residues in a given binding site will frequently not have been identified. Many of the known nucleotide affinity labels are limited in their usefulness because of their poor solubility, their low reactivity, or the bulkiness of their functional groups. We have synthesized two new adenosine nucleotide analogues, 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenosine 5′-monophosphate and 5′-diphosphate, and have determined their structures. These compounds are closely related to the adenine nucleotides, are water soluble, and are negatively charged at neutral pH. The bromoketo group is potentially reactive with most nucleophiles found in proteins (Hartman, 1977), and the dioxo group lends the possibility of reaction with arginine residues (Yankeelov, 1970; Riordan, 1973). Because of the location of the functional groups adjacent to the 6-position, the compounds might be expected to react with amino acid residues in the purine region of the adenine nucleotide binding sites of proteins. The analogous 5-(4-bromo-2,3-dioxobutyl) coenzyme A has previously been synthesized (although it was not fully characterized) and has been found to inactivate several enzymes that bind acetyl-CoA (Owens & Barden, 1978; Clements et al., 1979; Katiyar et al., 1982). In this paper, we present evidence suggesting that the new 6-[(4-bromo-2,3-dioxobutyl)thio]-6-deaminoadenine nucleotides react covalently at the catalytic site of rabbit muscle pyruvate kinase and at the ADP activating site of pig heart NAD-dependent isocitrate dehydrogenase.

† From the Department of Chemistry, The University of Delaware, Newark, Delaware 19711. *Received November 22, 1983.* This work was supported by NSF Grant PCM-8201969 and by U.S. Public Health Service Grant AM17552.

0006-2960/84/0423-3281$01.50/0