Thermodynamic, Kinetic, and Structural Basis for Recognition and Repair of 8-Oxoguanine in DNA by Fpg Protein from *Escherichia coli*†

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ABSTRACT: X-ray analysis does not provide quantitative estimates of the relative importance of the molecular contacts it reveals or of the relative contributions of specific and nonspecific interactions to the total affinity of specific DNA to enzymes. Stepwise increase of DNA ligand complexity has been used to estimate the relative contributions of virtually every nucleotide unit of 8-oxoguanine-containing DNA to its total affinity for *Escherichia coli* 8-oxoguanine DNA glycosylase (Fpg protein). Fpg protein can interact with up to 13 nucleotide units or base pairs of single- and double-stranded ribo- and deoxyribo-oligonucleotides of different lengths and sequences through weak additive contacts with their internucleotide phosphate groups. Bindings of both single-stranded and double-stranded oligonucleotides follow similar algorithms, with additive contributions to the free energy of binding of the structural components (phosphate, sugar, and base). Thermodynamic models are provided for both specific and nonspecific DNA sequences with Fpg protein. Fpg protein interacts nonspecifically with virtually all of the base-pair units within its DNA-binding cleft: this provides ∼7 orders of magnitude of affinity ($\Delta G° \approx -9.8$ kcal/mol) for DNA. In contrast, the relative contribution of the 8-oxoguanine unit of the substrate ($\Delta G° \approx -0.90$ kcal/mol) together with other specific interactions is <2 orders of magnitude ($\Delta G° \approx -2.8$ kcal/mol). Michaelis complex formation of Fpg protein with DNA containing 8-oxoguanine cannot of itself provide the major part of the enzyme specificity, which lies in the k_{cat} term; the rate is increased by 6−8 orders of magnitude on going from nonspecific to specific oligodeoxynucleotides.

On the basis of three-dimensional X-ray data, the structures of proteins, DNA, and RNA, as well as their conformational changes and additional protein−protein, RNA−RNA, and DNA−DNA interactions on complex formation, play important roles in the recognition of specific DNA and RNA sequences by proteins (*1*−*8* and references cited therein). However, X-ray structural analysis of protein−nucleic acid interactions does not provide quantitative estimates of the relative importance of molecular contacts or of the relative contributions of strong and weak, or of specific and nonspecific, contacts to the total affinity of an enzyme for DNA. X-ray analyses of sequence-specific enzymes with DNA have led to the concept, to some extent wrong, that specific contacts, such as pseudo-Watson−Crick interactions, provide high affinity for specific DNA sequences and that such interactions lead to high specificity and high efficiency in catalysis.

It is commonly supposed that enzymes recognizing specific ds[1] sequences cannot bind mononucleotides or short ss ODNs with high efficiency. There are few literature data on the quantitative evaluation of the relative individual contributions of thermodynamic (complex formation) and kinetic (reaction rate constant) steps of a catalytic process to the DNA affinity or to the specificity of enzymes. Nor have we found data on the quantitative evaluation of the relative contributions of specific and nonspecific interactions to DNA recognition by sequence-dependent enzymes, either at the complex formation step or at a later catalytic step. Only detailed quantitative analysis of the relative contributions of all the factors above can give a correct interpretation of the qualitative views of protein−DNA contacts obtained from X-ray structure analysis. Therefore, we developed new approaches to evaluate the relative contributions of essentially every nucleotide unit of DNA to an enzyme's affinity for long DNA molecules,

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[1] Abbreviations: AP-EN, apurinic/apyrimidinic endonuclease; Fpg, formamidopyrimidine or 8-oxoguanine DNA glycosylase; IC_{50}, concentration giving 50% inhibition of activity; ODN, oligodeoxyribonucleotide; oxoG, 8-oxoguanine; SILC, stepwise increase in ligand complexity; ss and ds, single- and double-stranded, respectively; topo I, human topoisomerase I; UDG, uracil DNA glycosylase.

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including various structural elements of the individual nucleotide units (reviewed in refs *1* and *2*). Analysis of molecular interactions between enzymes and long nucleic acids by stepwise increase in ligand complexity (SILC) has shown that complex formation, including formation of contacts between an enzyme and specific sequences, cannot provide the basis of either high affinity for DNA or specificity (for examples of repair, topoisomerization, integration, and restriction enzymes). Virtually all nucleotide units within the DNA-binding cleft interact with the enzyme, and high affinity is mainly (5−8 orders of magnitude) provided by many weak, additive interactions between the enzyme and various structural elements of many DNA nucleotide units (reviewed in refs *1* and *2*). The relative contribution of specific interactions to the total affinity for DNA is rather small, not exceeding 1−2 orders of magnitude. The specificity of enzyme action is provided by the enzyme-dependent DNA stages of adaptation to the optimal conformation and by catalysis: k_{cat} increases by 3−8 orders of magnitude on going from nonspecific to a specific DNA.

Escherichia coli 8-oxoguanine DNA glycosylase (Fpg protein) removes a number of modified DNA bases damaged by reactive oxygen species, including $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$. Oxidative damage to the cell, suggested as significant in both carcinogenesis and aging (*9*−*14*), includes premutagenic modifications of DNA bases, leading to the formation of 8-oxoG, thymine glycols, FapyGua, and FapyAde (imidazole ring-opened guanine and adenine, respectively). One of the most common of such DNA lesions, 8-oxoG, is repaired by Fpg protein (*15*−*21*). Unlike Fapy, 8-oxoG is both miscoding and mutagenic. During DNA synthesis in *E. coli*, the appearance of 8-oxoG results in G-C pair replacement by A-T (*22*−*25*). Fapy blocks the activity of DNA polymerases (*26*), thus causing cell death.

Fpg protein removes a number of purine lesions from DNA, but oxoG is believed to be its major physiological substrate (*27*, *28*). In addition to its *N*-glycosylase activity, this protein also has a nicking activity, cleaving both the 5′- and 3′-phosphodiester bonds at an AP site by successive β- and δ-eliminations, leaving both the 3′- and 5′-ends of the gap phosphorylated (*29*). Fpg protein also catalyzes excision of the DNA 5′-terminal deoxyribose phosphate, the principal product formed by the cleavage by AP endonucleases at abasic sites (*30*). Two distinct bifunctional oxoG-specific glycosylase β-lyases identified in *Saccharomyces cerevisiae* differ in their preference for the base opposite the 8-oxoG. One of them is identical to the OGG1 gene product (*31*). Recently, a human DNA repair glycosylase for the removal of oxoG (hOGG1, human yeast OGG1 homologue) has been characterized (*32*). The structure of the core catalytic domain of bifunctional hOGG1 bound to an oxoG-C-containing duplex oligonucleotide has been reported (*33*). In addition, the crystal structure of trifunctional MutM (Fpg protein) from *Thermus thermophilus* has been determined and a structure calculated of the enzyme complexed with DNA (*34*).

We have recently provided direct observation by stopped-flow kinetics of a number of the primary kinetic steps for Fpg protein (*35*). To elucidate the structural determinants of substrate specificity and mechanism action of Fpg protein quantitatively, we report on the use of a SILC method to probe the interaction of Fpg protein with a series of ss and ds nonspecific and specific ODNs and analyze the results using a thermodynamic model of nonspecific and specific DNA recognition.

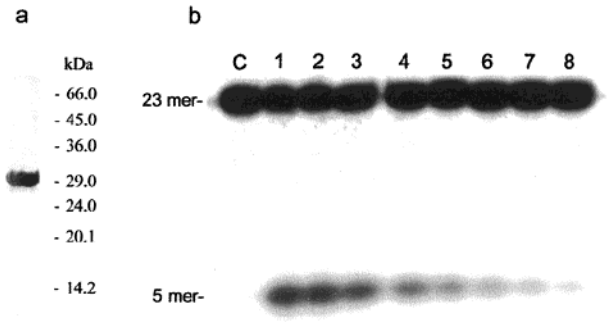

FIGURE 1: (a) SDS−PAGE of Fpg protein (11 μg) in a 15% gel; positions of markers are given on the right. The gel was stained with Coomassie R250. (b) Inhibition of Fpg protein activity in the case of ds 5′-[^{32}P]OG6 substrate (16 nM) by $d(pT)_{10}$ ODN (radioautograph): lane C (control), ds OG6 incubated alone; lanes 1−8, ds OG6 incubated in the presence of Fpg protein (0.7 nM) with $d(pT)_{10}$ at concentrations of 0 (lane 1), 0.05 (lane 2), 0.15 (lane 3), 0.3 (lane 4), 0.6 (lane 5), 1.2 (lane 6), 2.0 (lane 7), and 4.0 mM (lane 8), respectively.

MATERIALS AND METHODS

Materials. Reagents used were obtained mainly from Merck (Darmstadt, Germany) and Sigma (Poole, Dorset, U.K.).

Fpg Protein Purification. Electrophoretically homogeneous Fpg protein [(1−3) × 10^5 units/mg, Figure 1a] was purified from *E. coli* (JM105 cells containing the pFPG plasmid) by sequential chromatography on Q-Sepharose, S-Sepharose (Pharmacia), and Fractogel TSK HW-50 (Merck) and analyzed by SDS−PAGE as described (*36*).

Oligonucleotides. Synthesis, purification, and characterization of all ODNs including those listed below followed literature methods (*36*, *37*). The sequences of the 23-mer ODNs used are (**G*** is oxoG) as follows:

OG1, **G***CTCTCCCTTCCTCCTTTCCTCT;
OG2, C**G***TCTCCCTTCCTCCTTTCCTCT;
OG3, CT**G***CTCCCTTCCTCCTTTCCTCT;
OG4, CTC**G***TCCCTTCCTCCTTTCCTCT;
OG5, CTCT**G***CCCTTCCTCCTTTCCTCT;
OG6, CTCTC**G***CCTTCCTCCTTTCCTCT;
OG11, CTCTCCCTTC**G***CTCCTTTCCTCT;
OG20, CCTCTCCCTTCCTCCTTTC **G***TCT
OG21, CCTCTCCCTTCCTCCTTTCC**G***CT;
G6, CTCTC**G**CCTTCCTCCTTTCCTCT; and
G11, CTCTCCCTTC**G**CTCCTTTCCTCT.

ODNs containing the oxoG base in various positions are coded OG**N**, where **N** is the distance from the ODN 5′-end to the oxoG residue. ODNs containing unmodified G, instead of 8-oxoG, are indicated as G1−G11. Concentrations of ODNs were determined using calculated extinction coefficients (M^{-1} cm^{-1}) (*38*). The 23-mers containing oxoG were annealed to complementary sequences possessing dC opposite the oxoG residue. These complementary ODNs are indicated as C**N** (C1−C11), where **N** is the position of the oxoG (or nonmodified dG) base in the complementary strand.

Enzyme Activity Assay. One unit of Fpg protein can catalyze the excision of 1 pmol of oxoG from a duplex corresponding to OG11 in 1 min at 25 °C. The reaction mixture (10−60 μL) contained 50 mM Tris-HCl, pH 7.5,

Table 1: Affinity of Bases, Nucleosides, NMPs, dNMPs, and Other Small Ligands for Fpg Protein from *E. coli*[a]

ligand	IC_{50},[b] mM	K_I,[c] mM	ligand	IC_{50}, mM	K_I, mM
NaH_2PO_4	30	10			
deoxyribose	470	157	ribose	>400	>133
d(pR)[d]	25	8.3	pR	30	10
C base	145	48.3			
T base	140	46.7			
A base	60	20			
G base	55	18.3			
dTMP	25	8.3	UMP	20	6.7
dCMP	25	8.3	CMP	25	8.3
dAMP	10	**3.3**	AMP	26	8.7
dGMP	10	**3.3**	GMP	24	8.0
oxo-dGMP	2	**0.7**			

[a] In all experiments 0.7−1 nM Fpg was used. [b] Error in IC_{50} determination was 10−20%; average results of three to four measurements are given. [c] K_I values were calculated using IC_{50} values in all cases except oxo-dGMP, dGMP, and dAMP, for which K_I values were measured directly. [d] Deoxyribosephosphate.

50 mM KCl, 1 mM EDTA, 0.5 mg/mL BSA, 1 mM dithiothreitol, and 9% glycerol (v/v); concentrations of ODN(s) used were varied. Reactions were initiated by adding 0.1−1.0 enzyme unit (0.3−2 nM) for ds ODN substrate (or 10−20 units for ss ODN) and stopped after 2−20 min at 25 °C by the addition of aliquots (10−20 μL) of equal volumes of formamide buffer (80% formamide, 15% glycerol, and 10 mM EDTA). Reaction products were separated by 20% PAGE in the presence of 7 M urea (*39*). Gels were autoradiographed, and gel pieces corresponding to the [^{32}P]-ODN cleavage products were measured by Cherenkov counting. Initial rates were measured in the linear regions of the time courses and Fpg protein concentration curves.

Kinetic Parameters. K_M and V_{max} values were estimated using nonlinear regression analysis (*40*). K_I constants were determined using different concentrations of ODNs by direct nonlinear regression analysis (*40*). Values of IC_{50} were determined for various concentrations of ODN inhibitor (0.1−10 IC_{50}) at a ds OG6 substrate concentration of $2K_M$ (16 nM). Errors in IC_{50} were within 10−20%. For competitive inhibition (*40*, *41*), $IC_{50} = 3K_I$ when $S = 2K_M$, and errors in K_I were within 10−30%.

RESULTS AND DISCUSSION

Virtually any short specific or nonspecific ss or ds ODN can inhibit the Fpg protein reaction (*39*; Figure 1b). For OG6 as substrate, substrate and inhibitors are known to be competitive, so that K_I gives an estimate of binding affinity ($K_d = K_I$) of the Fpg protein's DNA-binding site for ODNs (*39*). As most of the short ODNs had relatively low affinities for Fpg protein, K_I values were calculated from the IC_{50} values using the equation corresponding to competitive inhibition, $IC_{50} = ([S]/K_M + 1)K_i$. Under the conditions used ([ds OG6] = $2K_M$) $IC_{50} = 3K_I$, a value that was observed experimentally with ss and ds ODNs (Tables 1−4).

Minimal Ligand of Fpg Protein. The Gibbs' free energy for complex formation can be taken as the sum of the $\Delta G°$ values for individual contacts (*43*)

$$\Delta G° = \Delta G_1° + \Delta G_2° + ... + \Delta G_n°$$

with $\Delta G_i° = -RT \ln K_d^i$, where K_d^i indicates the contribution of the individual contact (*40*, *41*). Hence, the overall K_d value for the formation of the Fpg protein:DNA complex is the product of the K_d values for individual contacts:

$$\Delta G° = -RT \ln K_d = RT \ln[K_d(1) \times K_d(2) \times ... \times K_d(n)]$$

Table 1 shows that the minimal ligands of Fpg protein are orthophosphate (P_i; K_I = 10 mM), dNMPs (K_I = 0.7−8.3 mM), and NMPs, with affinities for dNMPs and NMPs or for deoxyribosephosphate and ribosephosphate, being comparable (Table 1). Because Fpg protein shows increased affinity for oxo-dGMP (K_I = 0.7 mM), dGMP, and dAMP (Table 1), all structurally similar to oxo-dGMP, the site of increased affinity for dNMPs is most probably the Fpg protein active center, which removes oxoG from DNA. The affinity for oxo-dGMP is 4.7−11.8 times higher than for other nonspecific dNMPs (Table 1). The affinity for ribose

Table 2: Affinities of Deoxyribooligonucleotides (Oligos) and Their Derivatives for Fpg Protein from *E. coli*[a]

oligo	IC_{50},[b] mM	K_I,[c] mM	oligo	IC_{50}, mM	K_I, mM	K_M,[d] mM	oligo	IC_{50}, mM	K_I, mM
$d(pA)_2$	8.0	2.7	$d(pT)_2$	10.0	3.3		$d(pC)_2$	15.0	5.0
$d(pA)_3$	5.0	1.7	$d(pT)_3$	5.0	**1.7**				
$d(pA)_4$	3.9	1.3	$d(pT)_4$	4.0	1.3		$d(pC)_5$	2.5	0.83
$d(pA)_6$	2.3	0.77	$d(pT)_6$	1.75	0.58		$d(pC)_7$	1.5	0.5
$d(pA)_8$	0.75	0.25	$d(pT)_8$				$d(pC)_9$	0.5	0.17
$d(pA)_{10}$	0.25	**0.083**	$d(pT)_{10}$	0.35	**0.11**		$d(pC)_{10}$	0.3	**0.1**
$d(pA)_{11}$	0.25-	0.083	$d(pT)_{12}$	0.2	0.066		$d(pC)_{12}$	0.28	0.093
$d(pA)_{14}$	0.25	0.083	$d(pT)_{14}$	0.15	0.05		$d(pC)_{14}$	0.25	0.083
$d(pA)_{16}$	0.25	0.083	$d(pT)_{16}$	0.1	0.033				
$d(pA)_{20}$	0.20	0.07	$d(pT)_{20}$	0.1	0.033				
			$d(pT)_{23}$	0.09	0.030				
d[$A(pF)_2$][e]	8.0	2.7	d[$T(pF)_2$]	10.0	3.3		d[$(pF)_9$pT]	0.25	0.083
G11		**0.01**	OG11	0.007	**0.0021**	**0.001**			
G6	0.03	**0.01**	OG6	0.006	**0.002**	**0.001**			
C11		**0.006**	OG5			**0.0012**			
C3	0.03	0.01	OG4			**0.0011**			
C2	0.03	**0.01**	OG3			**0.001**			
C1	0.03	**0.01**	OG2	0.0015	**0.0005**	**0.0013**			
			OG1		**0.002**	**0.00075**			

[a] 0.7−1 nM Fpg protein was used in all experiments except for the determination of K_M values for ss specific ODNs (7−10 nM). [b] Error in K_M, K_I (shown in bold), and IC_{50} values determinations was 10−20%; average results of three to four measurements are given. [c] K_I values (nonbold) were calculated using IC_{50} values. [d] K_M values were from ref *39*. [e] (F) is a chemically stable analogue of deoxyribose (the tetrahydrofuran derivative).

Table 3: Affinities of Oligo-ribo-oligonucleotides and Their Duplexes for Fpg Protein from *E. coli*[a]

oligo or duplex	IC_{50},[b] mM	K_I, mM	oligo	IC_{50}, mM	K_I,[c] mM	oligo	IC_{50}, mM	K_I,[b] mM
$(pA)_2$	6.0	2.0	$(pU)_2$	8.0	2.7	$(pC)_2$	9.0	3.0
$(pA)_3$	5.5	1.8	$(pU)_3$	5.0	1.6	$(pC)_3$	6.0	2.0
$(pA)_4$	3.0	1.0	$(pU)_4$	3.0	1.0	$(pC)_4$	3.5	1.2
$(pA)_6$	2.0	**0.7**	$(pU)_6$	2.5	0.83	$(pC)_6$	1.6	0.53
$(pA)_8$	0.8	0.27	$(pU)_8$	0.52	0.17	$(pC)_8$	0.75	0.25
$(pA)_{10}$	0.5	0.17	$(pU)_{10}$	0.50	**0.16**	$(pC)_{10}$	0.6	**0.2**
$(pA)_{12}$	0.4	0.13	$(pU)_{11}$	0.50	0.16	$(pC)_{11}$	0.58	0.19
$(pA)_{16}$	0.4	0.13	$(pU)_{16}$	0.50	0.16	$(pC)_{12}$	0.58	0.19
$(pA)_{20}$	0.39	0.13				$(pC)_{15}$	0.5	0.17
$(pU)_8 \cdot (pA)_8$	0.19	0.063						
$(pU)_{10} \cdot (pA)_{10}$	0.06	**0.02**						
$(pU)_{14} \cdot (pA)_{14}$	0.06	0.02						
$(pU)_{16} \cdot (pA)_{16}$	0.05	0.017						

[a] 0.7−1 nM Fpg was used in all experiments. [b] Error in K_I (shown in bold) and IC_{50} determinations was 10−20%; average results of three to four measurements are given. [c] K_I values (nonbold) were calculated using IC_{50} values.

Table 4: Affinities of Mixtures and Duplexes of Oligonucleotides for Fpg from *E. coli*[a]

duplex	IC_{50},[b] μM	K_I,[c] μM	K_M,[d] μM	V_{max},[e] %
$d(pT)_2+d(pA)_2$	5900	1966		
$d(pT)_3+d(pA)_3$	4500	1500		
$d(pT)_4+d(pA)_4$	3300	1100		
$d(pT)_6+d(pA)_6$	800	267		
$d(pT)_8-d(pA)_8$	150	30		
$d(pT)_{10}-d(pA)_{10}$	20.0	**6.7**		
$d(pT)_{12}-d(pA)_{12}$	6.0	2.0		
$d(pT)_{14}-d(pA)_{14}$	1.5	**0.3**		
$d(pT)_{15}-d(pA)_{15}$	1.5	0.3		
$d(pT)_{16}-d(pA)_{16}$	2.0	0.67		
$d(pT)_{20}-d(pA)_{20}$	3.0	1.0		
G2−C2	0.21	0.07		
G11−C11	0.2	**0.067**		
OG1−C1	0.02	**0.0067**	**0.06**	**0.04**
OG2−C2	0.03	**0.01**	**0.016**	**0.44**
OG3−C3			**0.015**	**31.0**
OG5−C5			**0.01**	**10.0**
OG6−C6			**0.008**	**100**
OG11−C11		**0.006**[d]	**0.006**	**100**
OG20−C20			**0.006**	**0.1**
OG21−C21			**40.0**	**0.03**

[a] 0.3−0.7 nM Fpg protein was used in all experiments. [b] Errors in K_M, K_I (shown in bold), and IC_{50} value determinations were 10−30%; average results of three or four measurements are given. [c] K_I values (non-bold) were calculated using IC_{50} values. [d] K_M and V_{max} values were from ref *39*, and the value of K_d for OG11−C11 was from ref *45*. [e] V_{max} for OG11−C11 was taken as 100%; (C1−C21) oligos complementary to G1−OG21, respectively.

is very low (K_I = 157 mM). K_I values for all bases are in the range of 18.3−48.3 mM, and K_I values for P_i (10 mM; $\Delta G° = -2.84$ kcal/mol), 5′-deoxyribosephosphate (8.3 mM), and dTMP or dCMP (8.3 mM, $\Delta G° = -2.72$ kcal/mol) are comparable. Consequently, Fpg protein recognizes free dNMPs through interactions with all of its structural elements (base, sugar, and phosphate), the phosphate group making the major contribution. Although there is no absolute additivity expected of Gibbs free energies for different structural elements of various dNMPs, taking the product of K_I values for some bases (e.g., G; 20 mM), P_i (10 mM), and deoxyribose (dR) (157 mM) gives a net K_I value (2.9 mM) comparable to the K_I value determined for dGMP (3.3 mM; $\Delta G° \approx -3.4$ kcal/mol). The contributions of the interaction of Fpg protein with the dR moiety ($\Delta\Delta G° \approx -0.12$ kcal/mol) and of the nonspecific base N^0 ($\Delta\Delta G° \approx -0.54$ kcal/mol) of dNMPs with the Fpg lesion pocket may be approximately estimated from the ratios of K_d values for

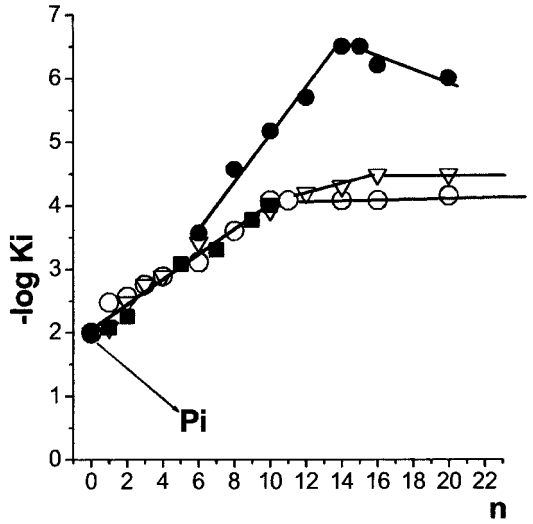

FIGURE 2: Dependencies of −log K_I for ss and ds deoxyribo-oligonucleotides versus length (n): $d(pT)_n$ (△), $d(pA)_n$ (○), $d(pC)_n$ (■), and $d(pT)_n-d(pA)_n$ (●). −Log K_I for orthophosphate corresponds to $n = 0$.

P_i to dpR or for dpR to dGMP, respectively (Table 1).

Additive Interaction of Fpg Protein with Nucleotide Units of Oligonucleotides. To assess the additivity of interactions of Fpg protein with ODNs, the data (Table 2) were analyzed as logarithmic dependencies of K_I (K_d) for $d(pN)_n$ versus the number of mononucleotide units (n) (Figure 2). The linear log dependencies for ss $d(pN)_n$ (for $0 \le n \le 10$, $n = 0$ corresponds to P_i) provide evidence of the additivity of $\Delta G°$ values for the interaction of 10 individual $d(pN)_n$ units with Fpg protein, consistent with published DNase footprinting studies where Fpg protein protects 9−10 nucleotides of ss DNA (*42*).

Values of f (1.56 ± 0.04), the increase in affinity of Fpg protein for various $d(pN)_n$ per unit increase in length, were evaluated from the slopes of the linear parts of these curves (Figure 2). The monotonic increases in K_d, reflecting interaction between enzyme and one unit of ss DNA, are equal to the reciprocals of these factors ($K_I = 1/f = 0.64 \pm 0.02$ M). Thus, the K_I values characterizing the affinity of Fpg protein for nonspecific dNMPs (3.3−8.3 mM) are ~80−200 times lower than the K_I (0.64 M) characterizing the enzyme interaction with any other nucleotide of an extended ODN. The interaction of Fpg protein with all units of $(pN)_n$ is additive, and the K_d (K_I) values for any ODN can be

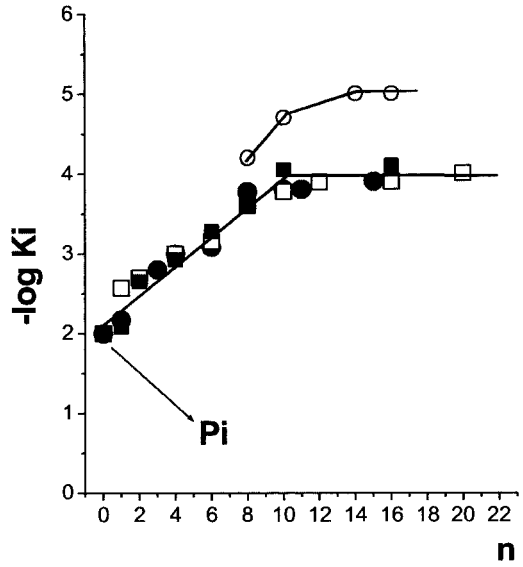

FIGURE 3: Dependencies of $-\log K_I$ for ss and ds ribo-oligonucleotides versus length (n): r(pA)$_n$ (□), r(pU)$_n$ (■), r(pC)$_n$ (●), and r(pA)$_n$−r(pU)$_n$ (○). −Log K_I for orthophosphate corresponds to $n = 0$.

obtained by multiplying K_d for the minimal ligand (P_i) by $K_d = 1/f$ for each of the mononucleotide units, according to a geometrical progression:

$$K_d[(pN)_n] = K_d[(P_i)][1/f]^n = K_d[(dNMP)][1/f]^{n-1} \quad (1 \leq n \leq 10)$$

The affinity of Fpg protein for nonspecific oligos d(pC)$_n$, d(pT)$_n$, and d(pA)$_n$ does not depend on the relative hydrophobicity of their bases (Figure 2). The ×a6 (1.56 ± 0.04) and K_d (∼0.64 M) values characterizing the weak interactions of Fpg protein with each nucleotide unit of different ss d(pN)$_n$ were nearly the same. Thus, Fpg protein essentially does not contact 9 of the 10 DNA bases, but mainly interacts with the sugar−phosphate backbone of the ligand. To confirm this and to probe the contribution of sugar−phosphate backbone structural units to the formation of weak additive contacts between Fpg protein and ODNs, we synthesized abasic oligomers, d[A(pF)$_2$], d[T(pF)$_2$], and d[(pF)$_9$(pT)], where F is a chemically stable analogue of deoxyribose with the base replaced by a hydrogen atom. The affinity of d(pN)$_3$ and d(pN)$_{10}$ for Fpg protein did not differ from that of the above trimers and d[(pF)$_9$(pT)], respectively (Table 2).

To estimate the contribution of the sugar moieties to binding, the affinities for (pN)$_n$ and d(pN)$_n$ were compared. The absolute K_I values for d(pN)$_n$ and (pN)$_n$ of the same length (n= 1−10; Tables 1−3) or slopes of log K_I versus n for corresponding d(pN)$_n$ and (pN)$_n$ (Figures 2 and 3) do not differ. The affinity of Fpg protein for various (pN)$_{10}$ ligands (K_I = 0.16−0.2 mM) is only 1.5−2 times lower than for d(pN)$_{10}$ (K_I = 0.083−0.11 mM). The ×a6 factor (1.52 ± 0.06) and K_d (∼0.66 ± 0.03 M) values for ss (pN)$_n$ ribo-oligonucleotides are effectively the same as for d(pN)$_n$.

The linear log dependencies for $1 \leq n \geq 10$ (Figures 2 and 3) indicate comparable efficiency of the interaction of Fpg protein with 9 of the 10 internucleotide phosphates of ss d(pN)$_{10}$ and the absence of strong contacts in the case of nonspecific ODNs. A ∼1.5-fold change in affinity on ss ribo- and deoxy-(pN)$_n$ elongation by one nucleotide unit (corresponding to a change in $\Delta G°$ of ∼ −0.26 kcal/mol) is lower than would be expected for strong electrostatic contacts (up to −1.0 kcal/mol) or hydrogen bonds (−2 to −6 kcal/mol) but comparable to values for weak hydrophobic, ion−dipole, and dipole−dipole interactions (*41*). Thus, the interaction of negatively charged internucleotide groups of nonspecific ODNs with the DNA channel of Fpg protein may use dipolar electrostatic interactions rather than electrostatic interaction of immediately contacting groups. The crystal structure MutM consists of two domains connected by a flexible hinge, with a large, electrostatically positive cleft lined by highly conserved residues between the domains (*34*). Therefore, the interaction is that of negatively charged internucleotide groups with the positively charged surface of the Fpg protein (i.e., typical of the interaction between oppositely charged surfaces of biopolymers).

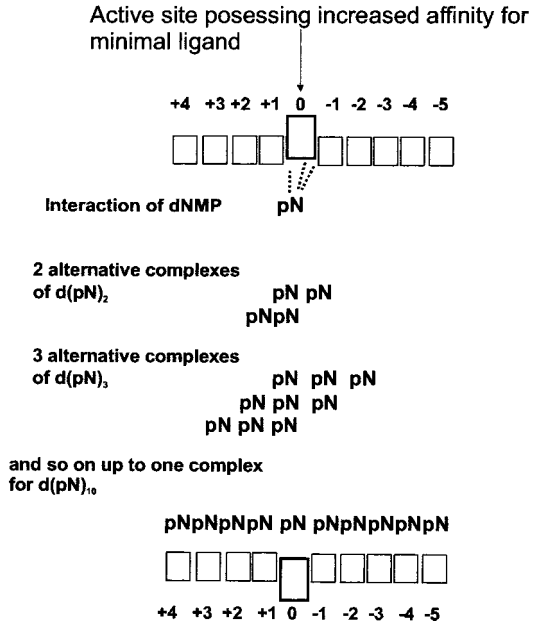

FIGURE 4: Schematic diagram of the DNA-binding site of Fpg protein. The DNA-binding site of the enzyme consists of two sets of 10 subsites: only one set of subsites interacting with cleaved strand, which is shown in the figure, contains a specific subsite (the *zero* subsite) demonstrating increased affinity for one specific or nonspecific nucleotide unit of DNA. Lengthening of nonspecific d(pN)$_n$ ($1 < n < 10$) leads to formation of several alternative and energetically comparable complexes of these oligonucleotides with different subsites on the enzyme.

The active center of the Fpg protein lies in the 5′-terminal region of the DNA-binding site about 5 nucleotides from the 5′ end of bound 10-mer ss ODNs (*42*). Considering the above data, the interaction of Fpg protein with nonspecific short ss ODNs can be described using Figure 4. All dNMPs interact with the lesion active center of Fpg protein through relatively strong nonspecific contact with their phosphate group and relatively weak nonspecific contacts with their bases and sugar moieties. ODNs containing two or more nucleotides can interact with Fpg protein through the formation of several alternative thermodynamically comparable complexes, the number of which increases with oligonucleotide length (n) (Figure 4). Finally, d(pN)$_{10}$ or (pN)$_{10}$ can form only one complex with the enzyme. All interactions of Fpg with 10 nucleotide units of ODNs, except one, are weak and additive.

To clarify the functional importance of the interaction between Fpg protein and ss ODNs, we compared the efficiency of complex formation of Fpg protein with nonspecific ss homo- and hetero-$d(pN)_n$ ligands with ODNs containing specific oxoG units. Of all nonspecific homo-$d(pN)_n$ analyzed, $d(pT)_{16-23}$ ($K_I = 30-33$ μM) showed the highest affinity. The affinities of various nonspecific ss hetero-$d(pN)_{23}$ containing one or several G bases in various positions (C1, C2, C3, C11, G6, and G11; Table 2) were 3.3−5.5 times higher than for $d(pT)_{23}$. Going from these hetero-$d(pN)_{23}$ to specific ss 23-mer ODNs with oxoG in different positions (OG1−OG11) led to an additional 4−30-fold increase in affinity (Table 2). The difference in affinity of the Fpg protein for specific and nonspecific ss ODNs was essentially the same as the difference in affinity for the complementary duplexes corresponding to them (see below). Thus, the introduction into ss ODNs of a specific oxoG, or even a structurally similar G base, can lead to the formation of additional contacts between Fpg protein and ss DNA. The functional significance of the interaction of Fpg protein with ss ODNs is that they are substrates (*39*), and K_M values measured for ss specific ODNs were equal or comparable to the corresponding K_I values (Table 2).

Affinity of Fpg Protein for Nonspecific DNA Duplexes. UDG melts ds $d(pN)_{10}$ partially and contacts both chains of such relatively short ODNs almost independently (*2*). In contrast, DNA polymerases, AP EN, and topo I interact with both base-paired DNA strands (*2*). However, the contribution of the second strand to the affinity of ds DNA for any enzyme is usually much smaller than that of the first one. A peculiarity of the behavior of topo I and DNA polymerases is the "assembly" and subsequent stabilization of correct duplexes for which the melting temperature (T_m) in solution is substantially lower than the reaction temperature (*2*).

Figure 2 and Table 4 show that the minimal ligand exhibiting duplex properties toward Fpg protein is $d(pT)_8$:$d(pA)_8$, for which the value of T_m (21 °C) (*43*) is close to the reaction temperature (25 °C). Calculated (*43*) T_m values of shorter duplexes are noticeably lower than the reaction temperature, so they behave under the reaction conditions as ss ODNs, not duplexes (Table 4). Thus, no effective stabilization of short duplexes is achieved by their interaction with Fpg protein. In contrast to ss ODN, a linear increase in log K_I for duplexes proceeds up to $n = 14$. The affinity of Fpg protein for duplexes is ~2 orders of magnitude higher than for ss ODN. The change in $d(pT)_n$:$d(pA)_n$ duplex affinity ($n = 8$) is described formally by the same algorithm as for ss ODN (see above), but the factor f increases to 2.22 ± 0.04. The ratio of these f factors (characterizing increase due to the addition of one base pair of the affinity for the first strand with that for the second strand) is 1.42 ±. 0.04, with corresponding K_d and $\Delta G°$ values of 0.7 ± 0.03 M and 0.21 ± 0.01 kcal/mol, respectively. Note that a single A-T and G-C pair formation in solution is characterized by $\Delta G°$ values of −1.2 to −1.9 and −2.0 to −2.8 kcal/mol, respectively (*2*).

Whereas the relative contribution of the first chain to the affinity is ~5 orders of magnitude [K_I for $d(pT)_{16-20} = 3.0-3.3 \times 10^{-5}$ M], addition of the second chain leads to an increase in affinity of only 2 orders of magnitude [K_I for $d(pT)_{15}$:$d(pA)_{15} = 3.0 \times 10^{-7}$ M]. Thus, the contribution of the second strand is less than that of the first.

The affinity of ds ODN for Fpg protein increases on elongation up to $n = 14$, again correlating well with 13−14 base pairs of ds ODN protection by Fpg protein against nuclease hydrolysis (*42*). Sometimes, the relative adaptability of DNA to a specific conformation depends greatly on the presence of the complementary chain (*2*). Fpg protein may not be able to effectively transform its own conformation and that of ss ODNs to optimal, which is supported by reports on the decrease of the maximal rate of substrate conversion at the transition from ds to ss specific ODNs (*39*). Thus, Fpg protein can interact effectively with only 10 nucleotide units of ss DNA, but what is the origin of the increase to 13−14 protected base pairs for ds ODNs (*42*)? The zinc finger used in duplex recognition by Fpg protein contains positively charged and hydrophobic residues at the ends of the loop and at its apex (*44*). In seeking modified bases, the hydrophobic residues of the zinc finger located in the major groove of DNA may highlight thymine, the hydrophobic methyl group of which lies in the DNA major groove (*44*). This could explain the increased affinity of the enzyme (at $n > 10$) for $d(pT)_n$ compared with $d(pA)_n$ (Figure 2). In addition, the affinity increase for ds ODNs going from $n = 10$ to 13 may be caused by additional duplex contacts with amino acid residues of the zinc finger.

Thermodynamic Model of Fpg Protein Interaction with Nonspecific DNA. The contributions of interactions of nonspecific (pG) nucleotide (~3.4 kcal/mol) and their structural elements with the Fpg lesion pocket are thus dR (−0.12 kcal/mol), base (−0.54 kcal/mol), and P_i (−2.84 kcal/mol). Because 12 phosphate groups of one strand of ds $d(pN)_{13}$ interact with Fpg through weak, additive contacts ($\Delta G° \approx -0.26$ kcal/mol), the summarized relative contribution of these 12 internucleotide phosphates is $\Delta G° \approx -3.1$ kcal/mol. A similar value of $\Delta\Delta G° \approx -3.4$ kcal/mol may be calculated from the ratio of the K_d values for dTMP and $d(pT)_{16-20}$ (Tables 1 and 2). Thus, all contacts of Fpg protein with the 13 nucleotides [including (pG) unit] of the cleaved strand of DNA provide a maximum $\Delta G°$ of −6.8 kcal/mol. From the ratio of f factors (equal to ~1.42, $\Delta G°$ ~0.21 kcal/mol) characterizing the difference in increase of affinity of ds compared to ss ODNs, the contribution of 13 nucleotide units of the second strand to the affinity of ds DNA may be estimated as $\Delta G° \approx -2.73$ kcal/mol, which is comparable to the $\Delta\Delta G°$ value (−2.8 kcal/mol) corresponding to the ratio of the K_d values for ss $d(pT)_{16}$ and ds $d(pT)_{16}$:$d(pA)_{16}$. The sum of all such maximal $\Delta G°$ values gives $\Delta G° \approx -9.6$ kcal/mol, which is comparable with $\Delta G° \approx -9.8$ kcal/mol calculated from the K_d value for nonspecific 23-mer hetero-ODNs containing G bases (6.7×10^{-8} M). From the model for the MutM:DNA complex and X-ray analysis of hOGG1 (*33*, *34*) both enzymes partially melt DNA, creating a sharp kink in the substrate. If such melting also occurs for *E. coli* Fpg protein, the types of interaction used with nonspecific DNA can be summarized as the thermodynamic model in Figure 5.

Contribution of Specific OxoG in Specific DNA to Its Affinity for Fpg Protein. The K_I values for duplexes corresponding to OG1 and OG2 ($V_{max} = 0.04-0.44\%$), very poor substrates (*42*), were determined by using them as inhibitors of Fpg protein (Table 4) versus a good substrate, ds OG6-C6 ($V_{max} = 100\%$). The K_d value for a good substrate (OG11-C11) was estimated as 0.6 nM from gel retardation

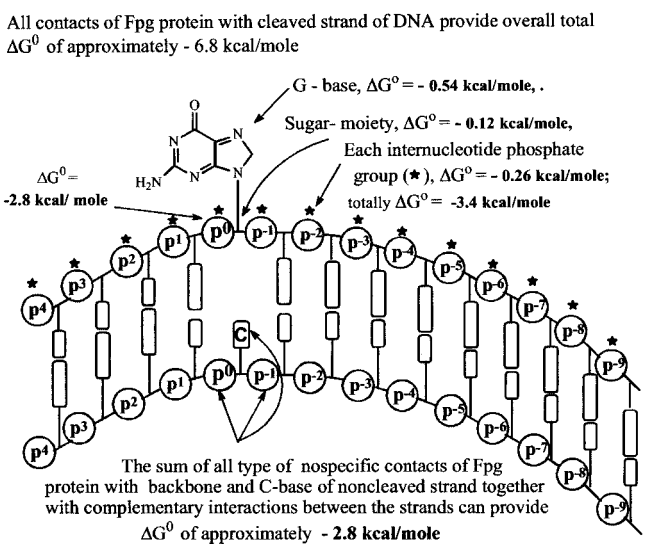

FIGURE 5: Thermodynamic model of the interaction of Fpg protein with nonspecific DNA. $\Delta G°$ values characterizing the various contacts between the enzyme and DNA containing G base are given. All types of nonspecific additive interactions of Fpg protein and DNA summed provide $\Delta G° \approx -9.8$ kcal/mol.

studies (*45*). Approximate affinities of Fpg protein for specific duplexes can be taken as literature K_M values (*39*), as these values are comparable with the corresponding K_I values (Table 4). The increase in affinity on going from nonspecific to specific ds DNA depends to some extent on the ODN sequence. The affinity of specific hetero-ODNs is only 50−75 times higher than for $d(pT)_{14-15}$−$d(pA)_{14-15}$, the strongest inhibitors among the homo-duplexes (Table 4). Substitution of G for oxoG in 23-mer hetero-ODNs increases affinity only 7−10-fold (transition from nonspecific to specific ss ODNs leads to a factor of 4−30). Interestingly, Fpg protein binds oxo-dGMP ~12 times more effectively than dTMP or dCMP and only 4−5 times better than dGMP or dAMP. Thus, the relative contribution of specific interactions of Fpg protein with oxoG base is comparable to the level of minimal ligands, ss $d(pN)_n$, and ds ODNs. This means that the contribution of specific and nonspecific interactions of different nucleotides of specific DNA to its total affinity for Fpg protein is close to additive, as found for two other repair enzymes UDG and AP-EN (*1*, *2* and references cited therein).

Thermodynamic Model of the Interaction of Fpg Protein with Specific DNA. The above algorithm adequately describes the changes of $K_d[d(pN)_n]$ when the minimal ligands are dTMP or dCMP. The experimental K_I for dAMP (~3.3 mM, Table 1) is somewhat higher than that calculated from the log dependence (~8.0 mM), as linearity for $d(pA)_n$ is observed only beginning with $d(pA)_2$ (Figure 2; Table 2). This may indicate increased affinity of the free nucleotide compared with the (pA) nucleotide unit for dinucleotides or longer ODNs. This may be a result of decreased hindrance to the interaction of free nucleotide, which can in principle bind in a greater range of orientations than can the internucleotide phosphate (which is constrained by its ligated 3′ and 5′ ends), especially if enzyme catalysis requires a degree of flipping out of the base or a specific change in structure of the (pA) unit for productive interaction of one link of longer $d(pA)_n$ with the lesion center of Fpg protein. As free oxo-dGMP also possesses maximal flexibility in its precise choice of optimal bound structure, the K_d value for this ligand may reflect the maximum possible affinity for an oxo-dGMP nucleotide unit within specific ds DNA. The total interactions of the Fpg protein lesion center with dGMP or dAMP have a K_d value of ~3.3 mM ($\Delta G° = -3.4$ kcal/mol), whereas all types of specific and nonspecific contacts between the Fpg protein pocket and oxo-dGMP (Table 1) may be estimated approximately as a K_d of ~0.7 mM ($\Delta G° = -4.3$ kcal/mol). As the interaction energies of different structural elements (base, phosphate, and sugar) of various dNMPs with the lesion center of Fpg protein are almost additive, the ratio (4.7) of the K_d values for dGMP and oxo-dGMP ($K_d \approx 0.21$ M; $\Delta\Delta G° = -0.90$ kcal/mol) gives an estimate of the relative contribution of the oxoG base for specific interaction of DNA with Fpg protein. Depending on the sequence of ligands used, going from nonspecific to various specific ss oxoG-ODNs increases affinity by 5−20-fold (Table 2), whereas the ratio of the K_I values for GMP and oxo-dGMP is only 4.7 (Table 1). Thus, a maximal enhancement of the affinity of specific ss ODNs compared with oxo-dGMP can be estimated as 4.2 ($K_d = 0.24$ M, $\Delta G° = -0.8$ kcal/mol). Stopped-flow experiments show that after formation of the Fpg protein:DNA primary complex, there is a conformational change of the enzyme, leading to an increase in enzyme affinity only for specific DNA (*35*). If Fpg protein can form additional contacts with internucleotide phosphate groups, such contacts can most probably be formed only after an Fpg protein-dependent change of conformation of specific ss DNA. Thus, $\Delta G° = -0.8$ kcal/mol may reflect a strengthening of previously formed weak additive contacts or the formation of new, stronger contacts between Fpg protein and internucleotide phosphates of ss ODNs.

The increase of the affinity on going from nonspecific to specific ds $d(pN)_n$ containing oxoG is 10−100-fold, depending upon the position of oxoG in the cleaved strand (Table 4). Thus, the maximal increase of the affinity of specific ds DNA compared with that for nonspecific ds DNA is characterized by $\Delta\Delta G° \approx -2.8$ kcal/mol. Therefore, an additional increase of affinity of specific DNA due to the presence of a second complementary strand may be characterized by $\Delta\Delta G° = -(2.8 - 0.9 - 0.8)$ kcal/mol $= -1.1$ kcal/mol. This affinity increase may be caused by several factors, including strengthening of the contacts formed between Fpg protein and ss ODNs in the absence of the second strand, a change in conformation of both strands, or even formation of additional contacts of Fpg protein with both first and second strands of ds DNA. The current data do not permit distinction of these possibilities. Overall, the interaction of Fpg protein with specific ds ODNs may be approximately described by the thermodynamic model of Figure 6. The efficiency of specific contact formation between Fpg protein and specific DNA does not exceed 2 orders of the total affinity: the relative contribution of nonspecific interactions to the total affinity is ~6−7 orders of magnitude.

From the model structure of the MutM:DNA complex (*34*), G and A bases can form all of the contacts revealed for oxoG and MutM. According to X-ray data for hOGG1, the most characteristic feature of oxoG, its 8-oxo-carbonyl function, is completely devoid of any interacting partner (*33*), and hOGG1 recognizes the urea system in oxoG through its N7-H. All other interactions between hOGG1 and oxoG are also

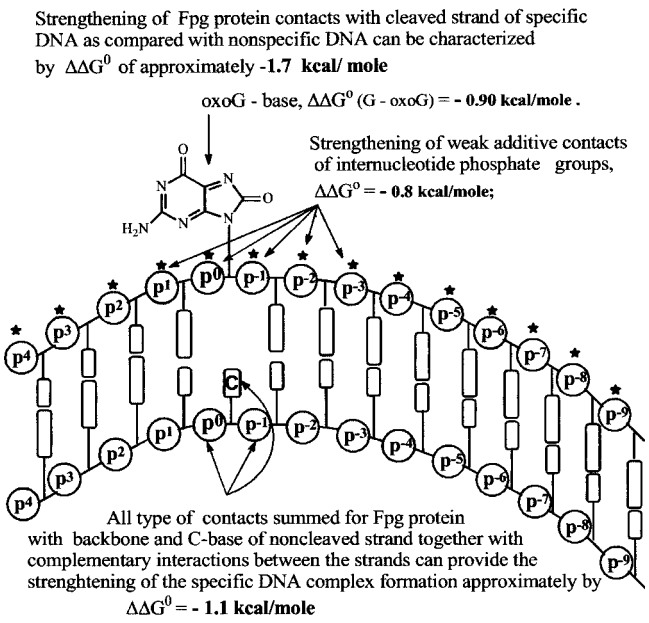

FIGURE 6: Thermodynamic model of the interaction of Fpg protein with specific DNA showing a strengthening of the enzyme contacts with the cleaved and noncleaved strands of specific DNA containing the oxoG base in comparison with nonspecific DNA (see Figure 5). The $\Delta\Delta G°$ value characterizing the change in all types of interactions summed at the transition from nonspecific to specific DNA is estimated to be about −2.8 kcal/mol.

nonspecific for damaged base. Thus, all contacts between oxoG of DNA and these enzymes are nonspecific and cannot provide high affinity for oxoG compared to G or A bases. This is consistent with our experimental demonstration for Fpg protein of a small difference in affinity for oxo-dGMP and dGMP, as well as for ss and ds ODNs containing oxoG and G bases (Tables 1−4).

Kinetic Factors: Reaction Rate and Specificity of Action of Fpg Protein. Complex formation of Fpg protein with DNA cannot explain high specificity in the enzyme action of Fpg protein. Previous studies showed that nonmodified C, T, G, and A bases can be removed from DNA by Fpg protein, but only at high enzyme concentrations and longer incubation times (10^4−10^7 times) than for specific DNA containing the oxoG base (*28*, *39*). Fpg cannot excise bases from nonspecific DNA with noticeable efficiency: the rate of nonspecific enzyme action decreases by 6−8 orders of magnitude (*28*, *39*). The catalytic stage appears to be significantly more sensitive to the DNA structure than the stage of complex formation between enzyme and DNA.

CONCLUSIONS

The present study shows for the first time that recognition by Fpg protein of specific DNA is additive in terms of Gibbs free energies. Fpg protein, like UDG, AP-EN, topo I, *Eco*R1 (*1*, *2*), and HIV integrase (*46*), interacts effectively not only with specific ss and ds ODNs but also with nonspecific ones. The efficiency of interactions of these enzymes with the individual structural components of each chain is usually comparable with the efficiency of these contacts with low molecular weight ligands containing the same nucleotides. In all these cases, including Fpg protein, the length of ss and ds $d(pN)_n$ at which log dependencies plateau correlates well with the relative sizes of the enzyme molecules. Usually, 30−40 kDa enzymes, including Fpg protein, "cover" one helix turn or 10 base pairs of DNA. The number of DNA links protected against nuclease hydrolysis within Fpg protein and other enzyme complexes also correlates well with *n* corresponding to the plateau of the log dependencies (*1*, *2*). Like other enzymes Fpg protein effectively forms contacts only with the internucleotide phosphate groups of ds DNA. The factor *f* (1.54) for Fpg protein (reflecting its interaction with one internucleotide phosphate) is comparable with *f* factors for other enzymes: 1.35, UDG; 1.45, AP-EN; 1.52, Klenow fragment; and 2.0, *Eco*RI (*5*, *6*). The relative contribution of the second strand to the affinity of Fpg protein for ds DNA is much lower than for the first, as for other enzymes analyzed (*1*, *2*).

All of these enzymes, including Fpg protein, are known as highly specific enzymes, capable of catalyzing conversions of specific DNA ∼4−8 orders of magnitude more effectively than nonspecific (*1*, *2* and references cited therein). The increase in affinity of specific ds ODNs compared with nonspecific does not exceed 1−2 orders of affinity for these enzymes: estimates are 2−5 times (AP-EN), 7−10 times (UDG), 50−70 times (HIV IN), 50−100 times (*Eco*RI), 200−250 times (topo I) (*5*, *6*), and ∼100 times (Fpg protein). Thus, the relative contribution of nonspecific interactions to the total affinity is ∼4−5 orders of magnitude greater than that of specific interactions. Complex formation cannot alone explain the specificity of enzyme action observed in in vivo experiments. All of the investigated enzymes, including Fpg protein, interact with noncognate RNA−RNA and RNA−DNA duplexes with affinities comparable to those for DNA−DNA duplexes, and the affinity for such complexes is still only 1−2 orders of magnitude lower than for specific DNA−DNA duplexes (*1*, *2*). However, the enzymes do not catalyze conversion of noncognate RNA−RNA and RNA−DNA duplexes even at saturating concentrations of these ligands. The data on X-ray analysis of MutM and hOGG1 (*33*, *34*) show that interaction of these enzymes with an oxoG-C base pair cannot differ very much from that with G-C or even other base pairs, and recognition of oxoG by these enzymes cannot provide very high affinity for specific compared with nonspecific DNA. The data indicate that all enzymes acting on lengthy DNA, including Fpg protein, can bind to the DNA of any sequence in the first stage and then "slide" to the site containing a specific lesion nucleotide unit or sequence (*1*, *2*). Here they stop due to the 5−250-fold increase in affinity for DNA and can change the conformation of the DNA sugar−phosphate backbone, partially or completely melting the DNA and destroying stacking interactions. The formation of pseudo-Watson−Crick hydrogen bonds between enzymes and specific DNA sites, such as have been elucidated by X-ray analysis for MutM or hOGG1, is most probably one of the final stages in the selection of specific substrates. Formation of just these bonds can promote the proper "docking" of specific bases into specific "pockets" of enzymes, accelerating their reactions by 4−8 orders of magnitude. On going from specific to nonspecific DNA, reaction rates in the case of all enzymes investigated so far, including Fpg protein, decrease by 6−8 orders of magnitude. It is not the stage of complex formation but rather the second (DNA adaptation to the optimal conformation) and third stages (the catalytic process, k_{cat}) that are the most important

steps in providing specificity for replication, repair, restriction, topoisomerization, and integration enzymes.

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