Kinetic and Photochemical Studies and Alteration of Ultraviolet Sensitivity of *Escherichia coli* Thymidine Kinase by Halogenated Allosteric Regulators and Substrate Analogues†

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ABSTRACT: The effect of various halogenated and nonhalogenated allosteric effectors on the sensitivity of *Escherichia coli* thymidine kinase to ultraviolet radiations (UV, 253.7 nm) was investigated. All naturally occurring dNTPs convert the monomeric form of the enzyme into the dimeric form which is less sensitive to UV inactivation. Whereas 5-iodo-2′-deoxycytidine triphosphate (IdCTP) and 5-iodo-2′-deoxyuridine triphosphate (IdUTP) enhance the UV inactivation of the enzyme, 5-bromo-2′-deoxyuridine triphosphate and 5-bromo-2′-deoxycytidine triphosphate exert a protective effect similar to that produced by the corresponding naturally occurring effectors, dTTP and dCTP. The enhanced UV inactivation by IdUTP is prevented totally by dTTP, but only partially by dCTP or dThd, whereas the enhanced sensitization by IdCTP is prevented almost totally by dCTP, partially by dTTP, and not at all by dThd. The UV sensitization of thymidine kinase by IdCTP appears to be at the regulatory site since a maximum saturation effect is observed, and the concentration required to exert a 50% maximal UV sensitization is similar to its K_m for enhancement of catalytic activity. When the enzyme was irradiated in the presence of either [2-^{14}C]IdUTP or [2-^{14}C]IdUrd, zone sedimentation analysis in sucrose density gradients showed the sedimentation coefficient of the radioactive labeled proteins to be the same, 3.8 S. Hence UV irradiation of the effector-induced dimer resulted in not only dissociation to the monomer, but also complete loss of catalytic activity. The substitution of an azido group for the 5′-OH group of 5-iodo-, 5-bromo-, 5-chloro-, or 5-fluorodeoxyuridine greatly decreased their affinity for thymidine kinase, and in addition the kinetics of inhibition changed from a competitive to a noncompetitive pattern. The presence of the azido moiety in the 5′ position of the halogenated nucleosides did not enhance the rate of UV inactivation of the enzyme.

Iwatsuki and Okazaki (1967) found *Escherichia coli* thymidine kinase formed a dimer in the presence of various allosteric regulators. Kinetic analysis showed dTTP was competitive with dThd,[1] but not with the phosphate donor or allosteric activators (Okazaki and Kornberg, 1964a). The effect of various halogenated deoxyribonucleoside triphosphates on *E. coli* thymidine kinase activity has been investigated, and IdCTP and BrdCTP were found to be more potent allosteric activators than the naturally occurring effector dCTP throughout the pH range of 5.2–9.3 (Voytek et al., 1971). Whereas IdUTP activated the enzyme at basic pH, an inhibitory effect comparable to dTTP was exerted below pH 6.5. The rates of UV inactivation of thymidine kinase, thymidylate kinase, and DNA polymerase are enhanced by substrate nucleosides or nucleotides which contain an iodine atom in the 5 position of the pyrimidine moiety (Voytek et al., 1972; Ku and Prusoff, 1974; Cysyk and Prusoff, 1972; Chen et al., 1976). The mechanism of UV sensitization of these enzymes involves a dehalogenation with formation of a uracilyl free radical (Rupp and Prusoff, 1964, 1965a,b) which subsequently interacts with and inactivates the sensitive biopolymer.

Azides have been shown to label specific antibody molecules (Fleet et al., 1969), specific binding sites of a membrane (Kiefer et al., 1970; Haley, 1975), or the active site of an enzyme (Pomerantz et al., 1975) when subjected to photolysis. Such sensitization of a macromolecule by an azide similarly involves an initial interaction of the ligand to a receptor, followed by the generation with radiations of a highly reactive ligand-derived species (free radical) that either covalently binds to the receptor site(s) or modifies it.

There is a need for agents that enhance radiation effects for chemotherapeutic objectives. As part of a program concerned with the development of such compounds, we have studied several aspects of UV-related sensitization of *E. coli* thymidine kinase. More specifically, several questions have been asked: (1) Do halogenated allosteric regulators sensitize the enzyme toward UV inactivation? (2) If such an effect does occur, is it specific for the regulatory site or is the catalytic site also affected? (3) What is the effect on the structure of the enzyme dimer following irradiation in the presence of halogenated regulators? Is the dimer "locked" into the dimeric form? (4) What structural features of substrate and allosteric regulatory analogues are required for UV sensitization? (5) What are the photoproducts of UV-irradiated halogenated allosteric regulators?

Experimental Procedure

Materials. E. coli thymidine kinase was purified by the procedure of Okazaki and Kornberg (1964a). [2-^{14}C]-Thymidine was obtained from New England Nuclear, dCTP, IdCTP, BrdCTP, IdUTP, BrdUTP, and dTTP from P-L Biochemicals, and [2-^{14}C]IdUrd from Schwarz/Mann. 5′-Azido analogues of various 5-halogenated deoxyuridine de-

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[1] Abbreviations used are: IdCTP, 5-iodo-2′-deoxycytidine triphosphate; IdUTP, 5-iodo-2′-deoxyuridine triphosphate; BrdUTP, 5-bromo-2′-deoxyuridine triphosphate; BrdCTP, 5-bromo-2′-deoxycytidine triphosphate; IdUrd, 5-iodo-2′-deoxyuridine; IdUMP, 5-iodo-2′-deoxyuridine monophosphate; dThd, deoxythymidine; DEAE, diethylaminoethyl; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid.

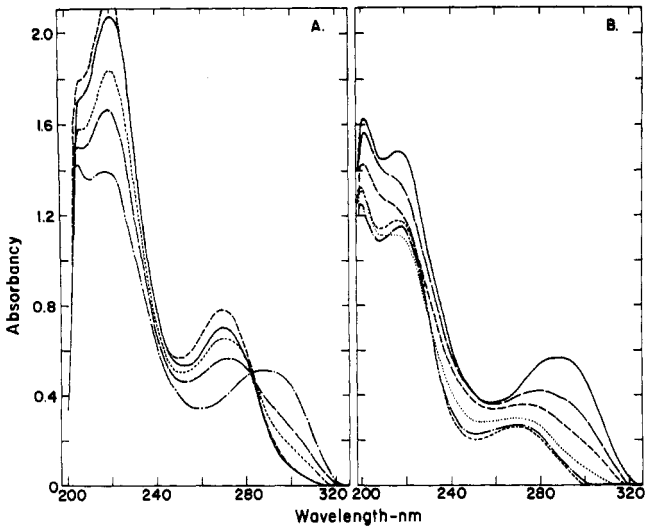

FIGURE 1: Ultraviolet spectra of irradiated and unirradiated 5-iodo-2′-deoxycytidine triphosphate in: (A) Tris-HCl (1 M, pH 5.2); the irradiation mixtures (2 mL) were irradiated for varying periods of time: (-·-) zero time control; (- -) 0.5 h; (···) 1 h; (—) 2.25 and 4 h; (- - -) after 4-h irradiation the solution was stored in the absence of light and 17 h later the spectrum was again determined; (B) phosphate buffer (1 M, pH 5.2), irradiated as in A: (—) zero time control; (— —) 0.5 h; (- - -) 1 h; (···) 2.25 h; (- - -) 5.5 and 6.2 h; (-·-) 17-h dark reaction.

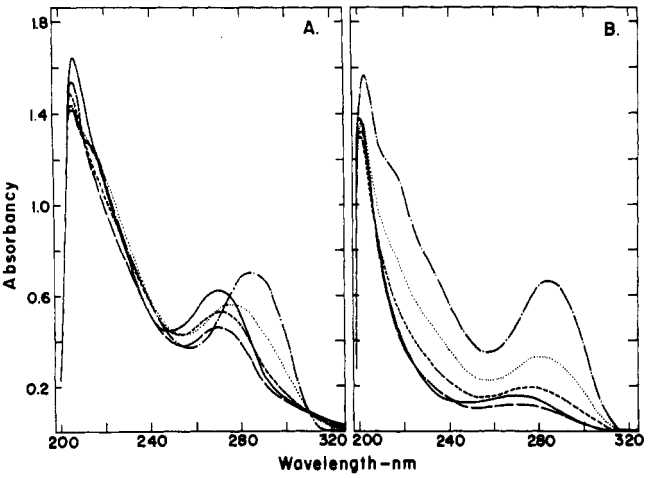

FIGURE 2: Ultraviolet spectra of irradiated and unirradiated 5-bromo-2′-deoxycytidine triphosphate in: (A) Tris-HCl (1 M, pH 5.2) and (B) phosphate buffer (1 M, pH 5.2). The irradiation mixtures (2 mL) were irradiated for varying periods of time: (-·-) zero time control; (···) 4.2 h; (- - -) 10.7 h; (- -) 21.8 h; (—) 23.6-h dark reaction.

rivatives were prepared by the procedure of Lin et al. (1976) and 5-iodo-2′-deoxy-6-azauridine was prepared by the procedure of Chen et al. (1976). All other chemicals used were reagent grade.

Enzyme Assay. The assay for thymidine kinase activity measured the conversion of [2-^{14}C]thymidine to [2-^{14}C]thymidylate by two procedures: (1) by adsorption onto DEAE-cellulose disks according to the method of Furlong (1963); (2) by thin-layer chromatography on cellulose Polygram cel 300 PEI/UV with either 0.5 M LiCl or 0.5 M LiCl-2 N acetic acid as the developing solvent. The composition of the enzyme assay mixture was reported previously (Voytek et al., 1971).

Spectral Analyses. Ultraviolet spectral analyses were conducted in a Beckman (Model 25) recording spectrophotometer.

Preparation of 5-Iodo[2-^{14}C]-2′-deoxyuridine Triphosphate. [2-^{14}C]IdUrd was converted enzymically to [2-^{14}C]IdUTP. The reaction mixture (0.3 mL) contained 20 μCi of a solution of lyophilized [2-^{14}C]IdUrd (sp act. = 48.5 mCi/mmol), 50 mM Tris-HCl (pH 7.8), 5 mM ATP, 5 mM $MgCl_2$, 0.075 mg of BSA, and 15.3 units of *E. coli* thymidine kinase. Following incubation at 37 °C for 1 h a 99% yield of IdUMP was obtained. IdUMP was purified by adsorption onto a DEAE-cellulose column in the bicarbonate form, followed by elution with triethylammonium bicarbonate as described previously (Prusoff and Chang, 1968). The procedure of Symons (1974) was used for the conversion of the formed IdUMP into IdUTP and its partial purification. The [2-^{14}C]IdUTP was further purified by DEAE-cellulose column chromatography as described above to remove the UV absorbing material which had been eluted together with [2-^{14}C]IdUTP from Whatman No. 1 chromatographic paper during the partial purification step. The purified product co-migrated with an IdUTP marker in four chromatographic systems: system A, 0.5 M LiCl, 2 N acetic acid; system B, 0.5 M LiCl; system C, 1-propanol-NH_4OH-water (55:10:35); and system D, isobutyric acid (660 mL)-0.1 M EDTA (2 mL)-water (300 mL) with concentrated NH_4OH to pH 3.7 and volume adjustment to 1000 mL with H_2O. Thin-layer MN-polygram cell 300 PEI/UV 254 plates were used with chromatographic systems A and B, and Whatman No. 1 chromatographic paper was used with systems C and D.

Irradiation of E. coli Thymidine Kinase. Prior to UV irradiation, the enzyme was passed through a coarse Sephadex G-50 column (1 × 40 cm, flow rate 0.3 mL/min) to remove β-mercaptoethanol which is necessary for stabilization during storage. The protein concentration in the irradiation mixture was maintained at 0.2 mg/mL by the addition of bovine serum albumin. UV irradiation of reaction mixtures that contained enzymes was carried out at 0 °C in 1.5-mL glass cups of 11 mm diameter. The source of UV light was a General Electric germicidal lamp (G15T8) having 86% of its total radiant intensity at 253.7 nm and less than 1% at other wavelengths below 300 nm, giving 320 erg mm^{-2} s^{-1} at 5 cm. The distance between the light source and the top of the solution to be irradiated was 5 cm unless mentioned otherwise. The radiation reaction mixture (0.3 mL) consisted of Tris-HCl (0.05 M, pH 7.8), 1 mM $MgCl_2$, 0.4 unit of the Sephadex-treated thymidine kinase, and various concentrations of the indicated allosteric regulators or substrate analogues. Except for IdCTP, the concentration of each dNTP is tenfold in excess of its K_m. All irradiation doses were corrected for the absorbance of the material being irradiated as described by Morowitz (1950).

Photochemical Studies. Photochemistry of compounds was performed under conditions similar to those described for the irradiation of thymidine kinase except that the temperature was increased to 20 °C, the buffer concentrations varied as specified in the figure legends, and the enzyme was not included.

Determination of Sedimentation Velocity Constants for Thymidine Kinase. Rate zonal sucrose density gradients (5–20%, w/v) were performed as described previously (Chen et al., 1976).

Results

Photochemistry of IdCTP. The effects of the composition of the reaction mixture on the photolysis of IdCTP and BrdCTP are shown in Figures 1 and 2, respectively.

Photolysis of IdCTP in Tris-HCl buffer (1 M, pH 5.2) resulted in a decrease in absorbance at 289 nm with the forma-

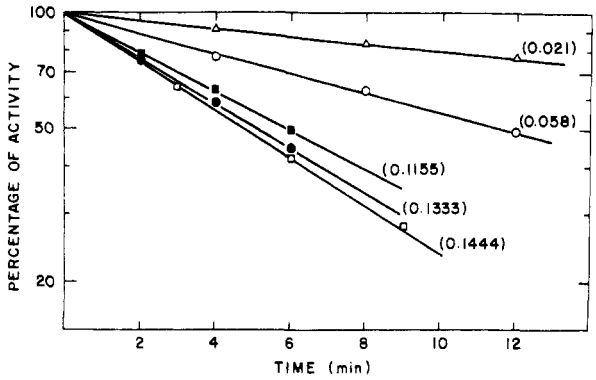

FIGURE 3: The effect of various halogenated allosteric regulators on the UV inactivation of thymidine kinase: (■) control; (●) 8 μM IdCTP; (□) 100 μM IdUTP; (○) 140 μM BrdCTP; (△) 350 μM BrdUTP. Numbers in parentheses are the first-order rate constants for inactivation of the enzymes (min^{-1}).

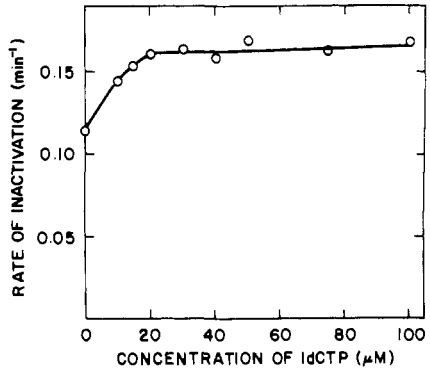

FIGURE 4: The effect of concentration of 5-iodo-2′-deoxycytidine triphosphate (IdCTP) on the rate of UV inactivation of thymidine kinase. The conditions of irradiation are described under Experimental Procedure. The rate of inactivation equals the first-order rate constant for inactivation of the enzyme (min^{-1}).

TABLE I: Effect of Naturally Occurring and Iodinated Allosteric Effectors on the UV Inactivation of *E. coli* Thymidine Kinase.[a]

Agent present	Rel rate of inactivation
None	1.00
dTTP (533 μM)	0.23
IdUTP (100 μM)	1.70
dCTP (270 μM)	0.59
IdCTP (270 μM)	1.40
IdUTP (100 μM) + dTTP (533 μM)	0.43
IdUTP (100 μM) + thymidine (533 μM)	1.03
IdUTP (100 μM) + dCTP (270 μM)	1.13
IdCTP (270 μM) + dCTP (270 μM)	0.73
IdCTP (270 μM) + thymidine (533 μM)	1.41
IdCTP (270 μM) + dTTP (533 μM)	1.03

[a] The conditions for UV irradiation and enzymic assay are described under Experimental Procedure.

TABLE II: Activation of Unirradiated and Irradiated *E. coli* Thymidine Kinase by the Allosteric Activator 5-Iodo-2′-deoxycytidine Triphosphate.[a]

Sample	IdCTP (mM)	Ratio of activation
Unirradiated	0	1
	0.1	3.08
	0.2	3.12
UV irradiated	0	1
	0.1	3.05
	0.2	3.13

[a] The *E. coli* thymidine kinase was UV irradiated in the presence of 0.1 mM IdCTP. After 53% of the activity had been destroyed, the IdCTP was removed by passing the reaction mixture through a coarse Sephadex G-50 column (1 × 40 cm) and the activity of both the unirradiated and UV-irradiated enzyme was assayed after the addition of either 0, 0.1, or 0.2 mM IdCTP. The ratio of activation = (thymidine kinase activity after addition of the allosteric activator IdCTP)/(thymidine kinase activity in the absence of added IdCTP).

tion of a new peak at 270 nm. The height of the peak at 270 nm increased 11% when the irradiated solution was stored in the dark for 17 h, a finding characteristic of reversible photohydration. Irradiation of dCTP under identical conditions resulted in a 10% decrease in its peak absorbancy at 270 nm, and upon discontinuation of irradiation, the original absorbance at 270 nm was fully recovered. Reversible photohydration of cytidine and its phosphate derivative has been reported previously (Shugar, 1960; Johns et al., 1965). Based on molar absorptivity of dCTP and IdCTP, the yield of transformation of IdCTP to dCTP is 88%. No detectable deamination to deoxyuridine triphosphate was found.

Irradiation of IdCTP in phosphate buffer and hence in the absence of a good hydrogen donor (Tris buffer) produced a relatively small peak at 270 nm, the transformation of IdCTP into dCTP under this condition being 32%. When irradiation of IdCTP was performed in water 27% transformation was obtained. Formation of dCTP when IdCTP is irradiated in water or phosphate buffer presumably involves abstraction of a hydrogen by a formed dCTP radical from another molecule of IdCTP.

The rate constant for the photolytic deiodination of IdCTP was determined to be 0.0158 min^{-1} and hence IdCTP is more photolabile than the corresponding uracil derivative, IdUTP. The rate constants for the photolytic deiodination of several iodinated compounds, in the presence of a hydrogen donor (e.g., Tris-HCl) under identical conditions, were determined recently, and found to be 0.0202 min^{-1} for 5-iodo-6-azadeoxyuridine, 0.0098 min^{-1} for 5-IdUMP, and 0.0267 min^{-1} for 5-iodo-6-azauracil (Chen et al., 1976). With each compound the substitution of the iodine atom by a hydrogen atom was found.

Photochemistry of BrdCTP. Photolysis of BrdCTP in 1 M Tris-HCl (pH 5.2) resulted in a decrease of the peak at 285 nm with the formation of a new peak at 270 nm (Figure 2A). The new peak decreased further upon continued irradiation; however, upon subsequent incubation in the dark this 270-nm peak increased in absorbance. This reversibility at 270 nm is a characteristic of the hydration photoproduct of dCTP which is readily converted in the dark to dCTP. Ninety percent and 23% of BrdCTP is converted into dCTP when irradiated in the presence of 1 M Tris-HCl (pH 5.2) (Figure 2A) and 1 M phosphate (pH 5.2) (Figure 2B), respectively. The rate constant for the photolytic debromination of BrdCTP was determined to be 0.0034 min^{-1}.

Halogenated Allosteric Regulators and UV Inactivation of Thymidine Kinase. Figure 3 shows the UV inactivation of the enzyme in the absence and presence of several halogenated allosteric regulators; they all appear to follow first-order reaction kinetics. Whereas IdCTP and IdUTP enhance the UV

TABLE III: Inhibition Constant of Various Nucleoside Analogues for *E. coli* Thymidine Kinase.[a]

Inhibitor	Type of inhibition	K_i (μM)
IdUrd	Competitive	7
5′-Amino-IdUrd	Competitive	240
5′-Azido-IdUrd	Noncompetitive	770
5′-Azido-BrdUrd	Noncompetitive	855
5′-Azido-CldUrd	Noncompetitive	1190
5′-Azido-FdUrd	Noncompetitive	2380
6-Aza-IdUrd	Competitive	1400

[a]The inhibition constants (K_i) were obtained from the double reciprocal plots of the effect of each inhibitor on thymidine kinase activity with thymidine as the variable substrate and ATP Mg^{2+} (6 mM) as the fixed substrate at pH 7.8. The K_m of thymidine was found to be 16 μM.

inactivation of the enzyme, BrdCTP and BrdUTP show apparent protection. Similar protection under these irradiation conditions is shown by dCTP and dTTP. The first-order rate constant for UV inactivation of the enzyme in the presence of dCTP (270 μM) is 0.068 min^{-1} and in the presence of dTTP (533 μM) it is 0.027 min^{-1}, whereas that for BrdCTP is 0.058 min^{-1} and that for BrdUTP is 0.021.

Table I summarizes the effect of naturally occurring and iodinated allosteric effectors on the UV inactivation of *E. coli* thymidine kinase.

IdCTP and UV Inactivation of Thymidine Kinase. The relationship of varying IdCTP concentrations on the rate of UV inactivation of thymidine kinase is shown in Figure 4. The hyperbolic curve is similar to a substrate saturation curve and indicates that the number of sites which can be sensitized by IdCTP is limited. The concentration of IdCTP required to attain 50% maximum enhancement of UV inactivation of thymidine kinase is 9 μM, which is similar to its K_m of 4 μM for augmentation of the catalytic activity of the enzyme, when evaluated as an allosteric effector (Voytek et al., 1971).

Irradiation of *E. coli* thymidine kinase in the presence of IdCTP caused total inactivation of the enzyme rather than destruction of only the regulatory site (Table II). If such a reaction had occurred only at the regulatory site, one would have expected the irradiated enzyme to have had a lower ratio of activation by IdCTP relative to that found with the unirradiated thymidine kinase.

Photoaffinity Labeling of E. coli Thymidine Kinase with an Iodinated Substrate and an Allosteric Regulator. The sedimentation patterns of the enzyme after irradiation in the presence of either the allosteric regulator [2-^{14}C]IdUTP or the alternate substrate [2-^{14}C]IdUrd are shown in Figure 5. Alcohol dehydrogenase from yeast was used as an internal standard. The ^{14}C-labeled proteins formed during irradiation of thymidine kinase with either labeled IdUrd or IdUTP had a sedimentation coefficient of 3.8. Voytek et al. (1971) reported that the native enzyme in the presence of IdUTP had an $s_{20,w}$ of 5.9, while in the absence of an allosteric regulator, the enzyme had an $s_{20,w}$ of 3.4.

Kinetics of Inhibition of Thymidine Kinase by Various Nucleoside Analogues. The effect of replacement of the 5 and 5′ moieties of thymidine with various groups on the activity of *E. coli* thymidine kinase is shown in Table III. The site which accommodates the 5′ position of the nucleoside at the active site of thymidine kinase appears to be quite restrictive. This is indicated by the change in the kinetics of inhibition from

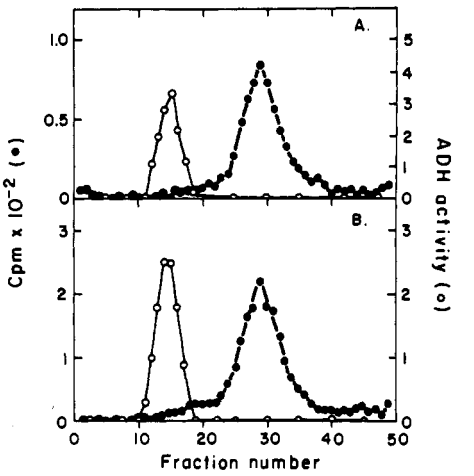

FIGURE 5: Effect of UV irradiation on the sedimentation coefficient of thymidine kinase when irradiated in the presence of either the alternate substrate IdUrd or the allosteric effector IdUTP. (A) *E. coli* thymidine kinase (38.5 U) was irradiated in the presence of 0.1 mM [2-^{14}C]IdUTP (5.4 μCi) until 80% of the enzyme activity was destroyed. The unbound radioactivity was removed by passing through a coarse Sephadex G-50 column (1 × 40 cm). The protein fraction (void volume) from the column was lyophilized, loaded onto a 5–20% sucrose gradient in 50 mM Tris (pH 7.8), and centrifuged in a Beckman SW-65 at 45 000 rpm for 17 h at 4 °C. Yeast alcohol dehydrogenase was used as an internal standard. Fractions were collected from the gradients. Each fraction was assayed for alcohol dehydrogenase activity and portions of each fraction were counted in a liquid scintillation counter with liquid fluor. (B) *E. coli* thymidine kinase (19 U) was UV irradiated in the presence of 0.098 mM [2-^{14}C]IdUrd (3.8 μCi) until 92% of the enzyme activity was destroyed. The irradiated enzyme mixture was analyzed as described in A.

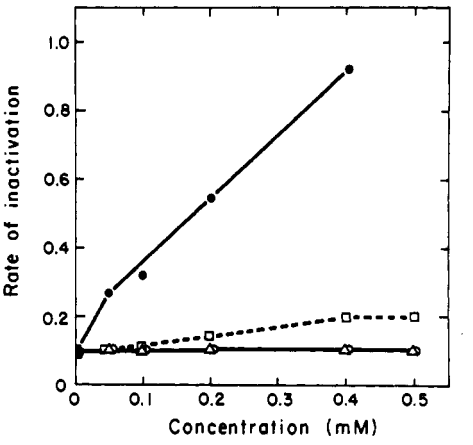

FIGURE 6: Effect of various 5′-azido-5-halogenated deoxyuridine derivatives on the UV inactivation of *E. coli* thymidine kinase. Details are described under Experimental Procedure: (●) 5′-azido-IdUrd; (□) 5′-azido-BrdUrd; (○) 5′-azido-CldUrd; (△) 5′-azido-FdUrd.

competitive to noncompetitive as well as the very large increase in K_i when the 5′-OH moiety is substituted by an azido group. However, replacement of the 5′-OH moiety with an amino group does not affect the type of inhibition but does decrease its binding affinity markedly as evidenced by the increase in K_i. Replacement of the carbon in the 6 position of IdUrd by a nitrogen (e.g., 6-aza-IdUrd) decreased greatly the affinity of the compound for thymidine kinase even though the 5′-OH moiety has been retained.

The Effect of the 5′-Azido Derivatives of 5-I-, Br-, Cl-, and FdUrd on the UV Sensitivity of E. coli Thymidine Kinase. Among these analogues only 5′-azido-IdUrd had a significant enhancement of UV inactivation of thymidine kinase (Figure 6). The rate of inactivation by the 5′-azido analogue of IdUrd

is similar but not greater than the rate of inactivation by IdUrd as reported by Cysyk and Prusoff (1972). Figure 6 also indicates that the nitrene radical, if produced from irradiation of the 5′-azido group, did not enhance the inactivation of the enzyme. Due to poor solubility of azido analogues, irradiation at higher concentrations could not be carried out in aqueous solution.

Discussion

The photolytic transformation of both halogenated allosteric regulators IdCTP and BrdCTP is presumed to proceed through a dCTP-free radical as the primary photoproduct which in the presence of a hydrogen donor (Tris buffer) abstracts a hydrogen atom and forms dCTP. Although dCTP was observed the nature of the major product formed during photolysis of IdCTP or BrdCTP when in a phosphate buffer and hence in the absence of a good hydrogen donor is unknown.

The apparent first-order reaction kinetics of photoinactivation of *E. coli* thymidine kinase is typical of the response of most enzymes to ultraviolet light (McLaren and Shugar, 1964) and indicates that only one photon is necessary for inactivation. Even though the brominated and iodinated dNTPs are potent allosteric regulators of thymidine kinase, only the iodinated analogues exert an enhancement of UV inactivation of the enzyme. The effect of halogenated nucleosides (5-I-, Br-, Cl-, and F-dUrd) on the UV inactivation of thymidine kinase had been investigated previously (Voytek et al., 1972), and here also only the iodinated analogues, IdUrd, enhanced the UV inactivation. The other three halogenated deoxyuridine substrate analogues neither protected nor sensitized thymidine kinase to UV inactivation. The enhanced UV inactivation by the iodinated nucleoside analogue and the absence of such an effect by the other halogenated nucleosides are related to the formation of a free radical in the pyrimidine moiety when IdUrd is irradiated (Cysyk and Prusoff, 1972; Rupp and Prusoff, 1965a,b).

E. coli thymidine kinase exists in the monomeric state and is readily dimerized in the presence of dCTP, dCDP, dTTP, or the corresponding halogenated analogues (Iwatsuki and Okazaki, 1967; Voytek et al., 1971). *E. coli* thymidine kinase is less sensitive to UV inactivation when converted into either the inactive dimer by dTTP or the activated dimer by dCDP (Cysyk and Prusoff, 1972). Thus, the apparent protection to UV inactivation afforded by BrdUTP or BrdCTP, which is similar to that produced by dTTP and dCTP, respectively, may be explained by their ability to also induce the monomeric enzyme into the dimeric form. The formation of an irreversible active dimer by "locking" it in that state by irradiation when in the presence of an allosteric activator (IdCTP) was not achieved (Table II); instead an inactive monomer was formed (Figure 5). Since IdCTP and BrdCTP have the same activating effect on the enzyme as the naturally occurring dCTP, the assumption is made that the halogenated analogues are probably bound to the same regulatory site as dCTP. The assumption that IdCTP is bound specifically to the activating regulatory site is supported by the data depicted in Figure 4. The K_m of IdCTP required for augmentation of catalytic activity is similiar to the concentration required to enhance UV inactivation to 50% of maximum. Ultraviolet sensitization of *E. coli* thymidine kinase by IdCTP probably is a consequence of the photolability of the carbon–iodine bond with formation of a dCTP-free radical when at the regulatory site. The number of UV sensitive sites in an IdCTP–enzyme complex is not known. The rate of dehalogenation of IdCTP is about 4.6 times the rate of photolytic debromination of BrdCTP which may explain in part the marked difference in their ability to sensitize thymidine kinase to UV irradiation.

Okazaki and Kornberg (1964b) reported that the inhibition by dTTP was noncompetitive with the phosphate donor or with the activator, but was competitive with thymidine. Since neither thymidine diphosphate nor thymidine monophosphate exerted an inhibitory effect, the authors suggested that the inhibitory site occupied by dTTP is not identical with that of thymidine, but rather overlaps or competes with it sterically because of its proximity. The experiment described in Table I was designed to differentiate the sites of photolytic action of halogenated allosteric regulators on the enzyme. The enhancement of UV sensitization exerted by IdCTP is decreased partially by the inclusion in the reaction mixture of either of the two naturally occurring regulators, dCTP or dTTP, but not by the normal substrate thymidine. Thus, even though IdCTP increases the binding affinity of dThd (K_m lowered), the nucleoside did not affect the augmented UV sensitization of IdCTP. The differential effect of dThd and dTTP on the enhanced UV sensitization by IdCTP supported the interpretation of Okazaki and Kornberg. Even though dTTP partially prevents the enhanced enzyme sensitization of IdCTP, binding at the same site by these two effectors is not implied, since the presence of these two allosteric regulators may merely readjust the population of dTTP- and of IdCTP-formed dimers. Whereas IdCTP converts the thymidine kinase monomer into a dimer that has augmented sensitization of UV radiation, dTTP converts the thymidine kinase monomer into a dimer that is markedly less sensitive to UV inactivation. Although both dTTP and dCTP decrease the relative rate of UV inactivation, dTTP inhibits the enhanced sensitization of IdUTP to a greater extent than that by IdCTP, and conversely dCTP inhibits more effectively than does dTTP the sensitization by IdCTP. Competition at the regulatory site as well as alteration in the population of enzyme dimers are important parameters to be considered in an explanation of these effects. Thus, the relative rates of UV inactivation of thymidine kinase by the uracil-containing dNTPs, dTTP and BrdUTP, are essentially identical (0.23 and 0.21), and are different from those of the cytosine-containing dNTPs, dCTP and BrdCTP (0.59 and 0.58), when comparison is made at concentrations tenfold in excess of their respective K_m.

Cysyk and Prusoff (1972) showed that IdUrd, which is an alternate substrate of thymidine kinase, sensitized the enzyme to inactivation by UV irradiation when at the catalytic site. Destruction of the regulatory site of this enzyme by the iodinated allosteric activator, IdCTP, also leads to a total loss of catalytic activity rather than the enzyme becoming "locked" into an activated dimer. In addition, the inactive enzyme dimer dissociates into an inactive monomer (Figure 5). The dissociation of the inactive dimer into the inactive monomer may imply that the monomer is thermodynamically more favorable than is the dimer form of the enzyme. The small difference between the sedimentation velocity of the inactive monomer (3.8 S) and the native monomer (3.4 S) may reflect a difference in the conformation of the two proteins.

The failure of the azido analogue of IdUrd to enhance the radiation sensitization beyond that of IdUrd may signify that the moiety of the 5′ position is not involved in the primary binding of the nucleoside to the enzyme (Table III) since it normally must be available for phosphorylation. Thus, even if the nitrene were produced, following UV irradiation of the azido nucleoside analogues, it may not be in sufficient proximity to a susceptible molecular linkage at the active site of the enzyme to afford the interaction required to effect enzyme

inactivation. Although most nitrenes have been reported to undergo intramolecular rearrangement rather than insertion into a nearby C-H bond (Lwowski, 1970), azido derivatives of metabolic substrates have been found to indeed covalently interact with the appropriate biopolymer (Fleet et al., 1969; Kiefer et al., 1970; Pomerantz et al., 1975; Haley, 1975).

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