Evidence That Deoxyribonucleic Acid Photolyase from Baker's Yeast Is a Flavoprotein†

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ABSTRACT: DNA photolyase purified from baker's yeast by affinity chromatography on UV-irradiated DNA noncovalently bound to cellulose and by chromatography on activated thiol-Sepharose 4B yields a single protein band having a molecular weight of 51 000 when analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The molecular weight, 53 000, determined by gel filtration was in good agreement. Upon denaturation of photolyase by heat or 8 M urea, flavin adenine dinucleotide (oxidized) was isolated from the mixture and identified by thin-layer chromatography and spectral analysis. In contrast to flavoproteins to which flavin adenine dinucleotide (oxidized) is bound which generally exhibit two absorbance maxima between 300 and 500 nm, photolyase has only one at 380 nm. These findings and the similar characteristics of the absorbance and emission spectra of native photolyase with those of flavoproteins in which the chromophore is considered to be the 4a,5-reduced flavin have led us to propose this configuration for the photolyase chromophore. The difference in properties of yeast photolyase compared to the one reported previously supports the idea that there are two photolyases in baker's yeast.

DNA photolyase is an enzyme found in many microorganisms, algae, plants, and some animals, including man (Rupert, 1975). The enzyme binds to cyclobutane dipyrimidine regions induced in DNA by far-UV irradiation, and upon illumination at higher wavelengths with near-UV or visible light, the cyclobutane ring is cleaved, resulting in collapse of the dipyrimidines to their original configuration.

Photolyases isolated from various sources apparently absorb photoreactivating light in different ways. The spectra of some photolyases have maxima in the near-UV or visible region and the enzymes emit fluorescence when excited in the native state (Eker & Fichtinger-Schepman, 1975; Eker, 1978; Minato & Werbin, 1971, 1972; Saito & Werbin, 1970), findings that suggest photoreactivating chromophores bound to these enzymes. In contrast, formation of an enzyme–substrate complex absorbing between 300 and 400 nm appears to be the active moiety in the case of the *Escherichia coli* enzyme (Wun et al., 1977).

In this study we report the purification of a yeast photolyase which has a chromophore absorbing and emitting at 380 and 460 nm, respectively, and which can be recovered from the denatured enzyme as FAD_{ox}.[1] Evidence is presented supporting the 4a,5-reduced configuration of FAD as the chromophore bound to the native enzyme.

Materials and Methods

Reagents. The FAD, FMN, and activated thiol-Sepharose 4B were from Sigma Chemical Co., and Calcon was from Eastman Kodak.

Photolyase Assay. Enzyme activity was measured by the transformation assay of Boatwright et al. (1975) and protein concentration by the procedure of Bradford (1976).

Polyacrylamide Gel Electrophoresis. $NaDodSO_4$–polyacrylamide (7.5%) slab gel electrophoresis was performed by the procedure of Weber et al. (1972) that was described for disc gel electrophoresis. Protein bands were stained with 0.2% Coomassie Brilliant Blue R-250 and scanned at 620 nm with a Joyce-Loebl microdensitometer. The molecular weight of photolyase was estimated by interpolation on a graph of the logarithm of the molecular weights of several standard proteins vs. the distance they migrated.

Sephadex G-150 Column Chromatography. A column, 1 × 105 cm, was packed with Sephadex G-150 that had been equilibrated with 0.05 M phosphate buffer (pH 7.5) containing 500 mM KCl, 1 mM EDTA, and 1 mM DTT. The photolyase and 0.5 mg each of several standard proteins dissolved in 0.5 mL of buffer were applied to the column. Elution was at a flow rate of 8 mL/h, and the 1-mL fractions collected were assayed for absorbance and photolyase activity.

Thin-Layer Chromatography. Thin-layer chromatography of flavins was performed on cellulose powder plates [MN Polygram Cell 300, Brinkman Instruments, Inc.; I.T.L.C. type S.A., Gelman Instrument Co.; Eastman chromogram sheets (silica gel)] using the solvent systems described in Table I (Maslowski, 1965). Flavins and the chromophore released from photolyase were detected by their fluorescence in 366-nm light.

Spectral Measurements. Excitation and emission spectra were recorded on a Perkin-Elmer Hitachi MPF-2A spectrofluorometer. Excitation spectra were corrected for by the Calcon–aluminum chelate method while the intensities of emission spectra were corrected by using standardized solutions of quinine sulfate and 3-aminophthalimide (White & Argauer, 1970). Absorbance measurements were made on Cary 14 and 219 spectrophotometers.

Amino Acid Analysis. This was done in the laboratory of Dr. J. Donald Capra at The University of Texas Health Science Center, Dallas, on a Durrum D-500 amino acid analyzer. Proteins were hydrolyzed for 18 h in 6 N HCl at 110 °C. Cysteine was determined as *S*-(carboxymethyl)cysteine (Crestfield et al., 1963) and tryptophan by the addition of 3-(3-indolyl)propionic acid to the acid hydrolysate (Gruen & Nicholls, 1972).

Purification of Photolyase. The purification was essentially that of Madden & Werbin (1974) and Boatwright et al. (1975)

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[1] Abbreviations used: FAD and FAD_{ox}, flavin adenine dinucleotide and its oxidized moiety; FMN, flavin mononucleotide; HSEtOH, 2-mercaptoethanol; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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Table I: Chromatographic Identification of the Flavin Bound to DNA Photolyase[a]

	R_f						
	MN Polygram Cell 300				silica gel		I.T.L.C.
sample	A	B	C	D	A	B	type S.A., C
extract of heated enzyme	0.12	0.44	0.46	0.46	0.088	0.57	0.74
FAD	0.12	0.44	0.48	0.46	0.088	0.59	0.73
FMN	0.22	0.58	0.51	0.57	0.18		0.70

[a] The purified enzyme (0.6 mg/mL) was dialyzed and heated for 5 min at 100 °C. Following centrifugation, 50–100-μL amounts of the supernatant were run along with authentic samples of FAD and FMN (5 μL of a 0.01% solution). The solvent systems used were the following: A, butanol-acetic acid-water (4:1:5, upper layer); B, butanol-acetic acid-water (4:3:3); C, 5% NaH_2PO_4; D, butanol-pyridine-water-acetic acid (3:3:3:1).

with the following modifications. Enzyme eluted from the phosphocellulose column between 0.37 and 0.47 M KCl was concentrated and dialyzed against buffer A (50 mM Tris, 20 mM potassium phosphate, 1 mM EDTA, 1 mM HSEtOH, and 200 mM KCl, pH 7.5). A UV-irradiated DNA–cellulose column (2.6 × 10 cm) was prepared (Minato & Werbin, 1971) and preequilibrated with buffer A. The dialyzed enzyme fraction was applied to the column and washed with 100 mL of buffer A. Elution was conducted with 400 mL of the same buffer, using a linear gradient of KCl between 0.2 and 0.8 M. The flow rate was 37 mL/h. Active fractions, eluted between 0.3 and 0.4 M KCl, were pooled, and glycerol was added to provide a 5% concentration. The material was concentrated in an Amicon Diaflo ultrafiltration concentrator using a PM-10 membrane and dialyzed against buffer B (50 mM Tris-HCl, 20 mM potassium phosphate, 1 mM EDTA, 2 mM DTT, 5% glycerol, and 250 mM KCl, pH 7.50). The enzyme fraction was applied to a second UV-irradiated DNA–cellulose column (2 × 5 cm) equilibrated with buffer B. The column was washed with 20 mL of buffer and eluted with a linear gradient of 0.25–0.7 M KCl in buffer B. The flow rate was 20 mL/h. Active fractions were concentrated and then dialyzed against buffer B containing 0.5 M KCl. The enzyme was stored in small vials at −80 °C.

Homogeneous photolyase could be isolated in another way. The concentrated enzyme from the first affinity chromatography (3.7 mg of protein per 4.7 mL) was dialyzed against a deaerated solution of 0.05 M Tris-HCl, pH 7.5, containing 500 mM KCl and 1 mM EDTA and then applied to a column (0.9 × 13 cm) of activated thiol-Sepharose 4B. The column was washed with 10 mL of buffer, and then the enzyme was eluted with the same buffer containing 10 mM DTT. The flow rate was 5.5 mL/h, and 1-mL fractions were collected. Active fractions were pooled and dialyzed against 0.05 M phosphate buffer, pH 7.5, containing 500 mM KCl, 1 mM EDTA, and 1 mM DTT and stored at −80 °C.

Results

Homogeneity of Photolyase. Because previous findings implicated an essential SH group on photolyase, the last step in the purification procedure, a second affinity chromatography on UV-irradiated DNA, was replaced by a covalent chromatography on activated thiol-Sepharose 4B. Enzyme purified this way stained as a single band when analyzed by electrophoresis on $NaDodSO_4$–polyacrylamide gels (Figure 1). The molecular weight determined by this technique was 51 000 ± 1000, a value that agreed with the estimate of 53 000 ± 1000 obtained by Sephadex G-150 gel filtration. For this analysis, photolyase in the column eluates was assayed enzymatically. Other batches of enzyme purified by two affinity chromatographs on UV-irradiated DNA had identical properties with the batch purified on activated thiol-Sepharose.

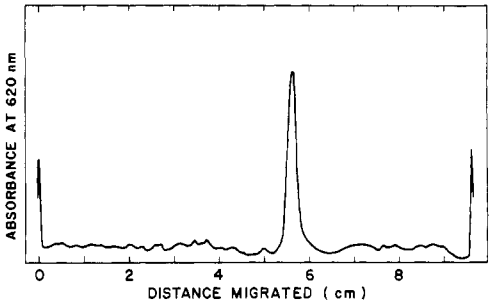


FIGURE 1: Densitometer scan of purified DNA photolyase run on a $NaDodSO_4$–polyacrylamide (7.5%) slab gel. The enzyme (30 μg) was dialyzed against 0.01 M Tris-HCl, pH 6.8, and then heated in a boiling water bath for 2 min after the addition of $NaDodSO_4$ to 1% and 2-mercaptoethanol to 0.1%.

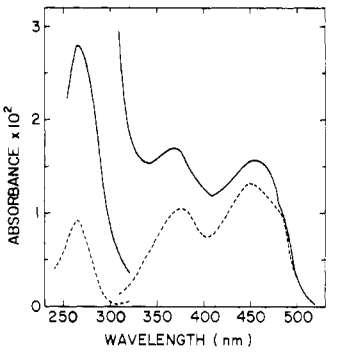


FIGURE 2: Absorption spectra of the supernatant fraction of heat-denatured DNA photolyase (—) and that of authentic FAD_{ox} (- - -). The enzyme solution (0.1 mg/mL) was dialyzed against 0.05 M phosphate buffer, pH 7.5, and then heated in a boiling water bath for 5 min. Following centrifugation, the absorbance measurements were made on the supernatant. Values noted between 250 and 320 nm have been reduced by 0.2.

Recovery of FAD_{ox} from Denatured Photolyase. Purified enzyme, 0.6 mg/mL, was extensively dialyzed and then heated for 5 min at 100 °C. The precipitate was removed by centrifugation at 10000*g* for 10 min, and 5–100-μL portions of the supernatant were examined by thin-layer chromatography. The data in Table I show that a substance in the supernatant had the same R_f as authentic FAD_{ox} in three chromatographic systems. Absorbance, excitation, and emission spectra (Figures 2 and 3) of the released material support its identification as FAD_{ox}. The high absorbance at 266 nm of the substance released from the enzyme relative to its absorbance maxima at 370 and 454 nm probably reflects some impurities or degradation products in the denatured mixture.

When the enzyme was denatured by an alternate procedure, addition of 8 M urea, identical spectrophotometric results were obtained.

Besides the usual protein maximum at 275 nm in the absorbance spectrum of the purified native enzyme, there was

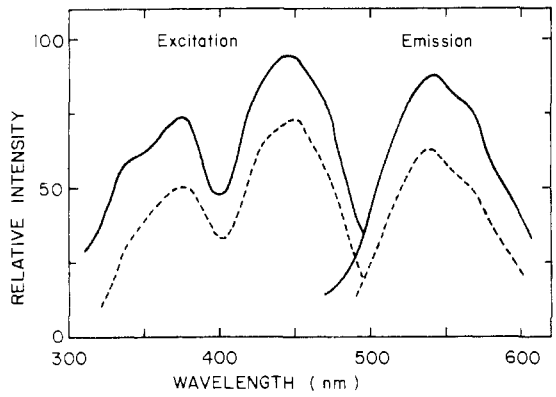


FIGURE 3: Excitation and emission spectra of the supernatant fraction of heat-denatured DNA photolyase (—) and authentic FAD_{ox} (---). The enzyme was treated as described in the legend to Figure 2. Excitation was monitored at 520 nm, and emission was excited by 380-nm light.

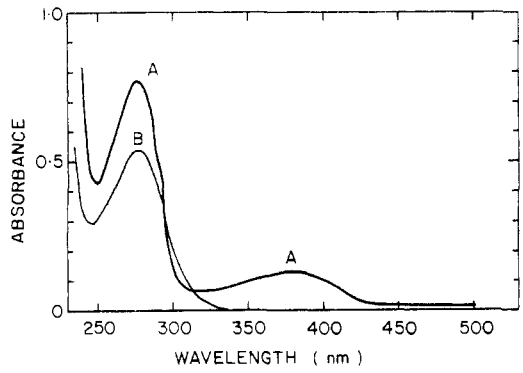


FIGURE 4: Absorption spectra of native (A) and denatured (8 M urea) (B) DNA photolyase. The denatured enzyme was dialyzed against 8 M urea prior to the absorbance measurements.

a maximum at 380 nm. The latter was eliminated when the enzyme was treated with and then dialyzed against 8 M urea (Figure 4).

Molar Ratio of FAD_{ox} to Native Photolyase. Purified photolyase was dialyzed against 0.05 M potassium phosphate buffer and then denatured in 8 M urea. The absorbance and emission spectra of the solution were determined, as were those of authentic FAD_{ox} treated similarly. The concentration of FAD_{ox} was estimated from its extinction coefficient at 450 nm, 11.3×10^3 mol^{-1} (unchanged in 8 M urea), or from the intensity of its fluorescence emission at 520 nm in 8 M urea. In the latter case the intensity was 2.4 times higher than it was in the absence of urea. Values of 0.85×10^{-6} and 0.92×10^{-6} M were obtained by these two procedures. From the average of these values and the protein concentration of photolyase after it was denatured in urea, 1.0×10^{-6} M, the calculated molar ratio of FAD_{ox} to photolyase was 0.83. When the enzyme was heat-denatured, the ratio was 0.74.

FAD_{ox} Is Not the Chromophore in Native Photolyase. Many enzymes requiring noncovalently bound FAD_{ox} as a cofactor exhibit two absorbance peaks, one at ~370–390 nm and a second with somewhat higher absorbance at 455–462 nm (Ghisla et al., 1974). These values are close to those for solutions of pure FAD_{ox} (380 and 450 nm, Figure 2). The absorbance maxima are reflected in the excitation spectra of many of these enzymes. The same enzymes exhibit emission maxima between 520 and 530 nm.

In contrast, native photolyase shows one absorbance and excitation peak at 380 nm (Figures 4 and 5) and a blue-shifted emission peak at 460 nm. Only after denaturation by heat or urea do the two absorbance maxima characteristic of FAD_{ox} appear (Figure 2).

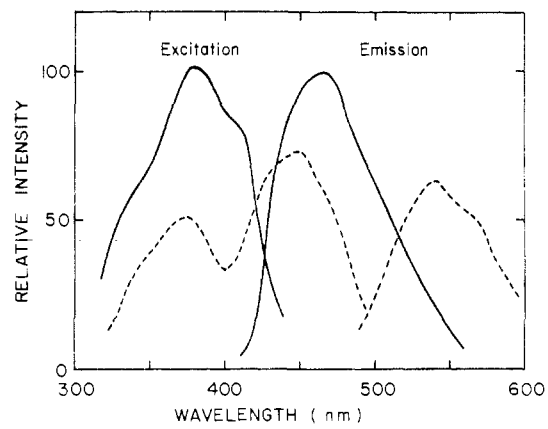


FIGURE 5: Excitation and emission spectra of native DNA photolyase (—) and authentic FAD_{ox} (---). Excitation was monitored at 460 nm, and emission was excited by 380-nm light.

Table II: Comparison of Amino Acid Compositions of Yeast, *E. coli*,[a] and Orchid[a] DNA Photolyases

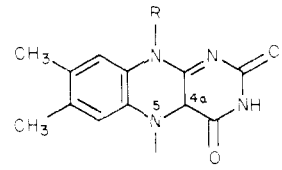
amino acid	yeast: amino acid residues/ mol of protein	yeast: % of total no. of residues	*E. coli*, % of total no. of residues	orchid, % of total no. of residues
Asp	42	8.7	12.8	11.2
Thr	21	4.3	4.3	4.2
Ser	70	14.5	4.3	2.1
Glu	62	12.8	12.8	12.5
Pro	16	3.3	4.3	4.2
Gly	72	14.9	8.5	10.4
Ala	33	6.8	8.5	8.3
Val	21	4.3	8.5	8.3
Met	14	2.9	2.1	2.1
Ile	19	3.9	6.4	6.2
Leu	29	6.0	8.5	8.3
Tyr	16	3.3	2.1	3.3
Phe	15	3.1	2.1	6.2
His	7	1.5	2.1	2.1
Lys	24	5.0	6.4	6.2
Arg	17	3.5	6.4	4.2
Cys	2	0.4	0	0
Trp	4	0.8	0[b]	0[b]
total	484	100	100	100

[a] Snapka & Fuselier (1977). [b] Analysis uncertain.

Amino Acid Analyses of DNA Photolyase. The amino acid composition of yeast photolyase is given in Table II as the number of amino acid residues per mole of protein and as the percentage of the total number of residues. The latter values are compared with those for the *E. coli* and orchid photolyases. The presence of cysteine and tryptophan in the yeast enzyme as well as a significantly higher glycine and serine content distinguishes it from the two other enzymes.

Discussion

Based on the following considerations, we propose that the chromophore noncovalently bound to photolyase is a 4a,5-reduced FAD. When photolyase is denatured by heat and



4a,5-reduced FAD

Table III: Absorption and Fluorescence Emission Maxima of Photolyase and Several 4a,5-Reduced Flavoproteins[a]

flavoprotein	coenzyme	absorption max (nm)	emission max (nm)	ref and comments
D-amino-acid oxidase C_{4a}-$CH_2C_6H_5$ adduct	FAD	365 320 sh	520	Ghisla et al. (1974)
4a-propyl-4a,5-dihydro-riboflavin 5'-monophosphate bound to apoflavodoxin of *P. elsdenii*	FMN	384	*b*	Scola-Nagelschneider et al. (1976)
bacterial luciferase intermediate II in oxidation of $FMNH_2$ by O_2	FMN	372	485	Becvar et al. (1978), Hastings et al. (1973); II breaks down at 20 °C to yield FMN_{ox} in the presence of oxygen
chromophore B of photoexcitable luciferase	FMN	375	495	Tu & Hastings (1975); irradiation of enzyme reconstituted with B yields FMN_{ox}
DNA photolyase	FAD	380	460	

[a] While there is good evidence for the 4a,5-reduced configurations of these flavoproteins, it has not been proved yet. [b] Not measured.

the mixture centrifuged, FAD_{ox} can be recovered from the supernatant. It can be recovered also by dialysis of the enzyme denatured by 8 M urea. Therefore, the chromophore must be structurally similar to FAD_{ox}, but it is not FAD_{ox} because in the spectra of those flavoproteins in which this flavin is noncovalently bound there are generally two absorption maxima, one between 447 and 460 nm and the other between 355 and 390 nm (Ghisla et al., 1974); but there is only one at 380 nm in the spectrum of native photolyase. In Table III there is a comparison of absorption and fluorescence emission maxima for photolyase and several proposed 4a,5-reduced flavoproteins culled from the literature. The presence of a single absorption maximum in these spectra supports the idea that the photolyase chromophore is a 4a,5-reduced flavin. It is of interest also that the chromophore in both luciferase flavoproteins (Table III) is converted, in one case by heating in the dark at 20 °C and in the other by light and oxygen, to oxidized FMN. The arguments used by Tu & Hastings (1975) to support the 4a,5-reduced flavin structure for chromophore B in photoexcitable luciferase are applicable to the photolyase chromophore also. Some 1,5-reduced flavoproteins exhibit one maximum and others two maxima in their absorption spectra; hence, this configuration cannot be completely ruled out for the chromophore.

Crystallographic data indicate that in reduced flavins there is bending along the N_5–N_{10} axis giving rise to a "butterfly" structure (Kierkegaard et al., 1971). The wide variation in the absorbance and fluorescence properties of reduced flavoproteins may reflect the extent that this nonplanarity is preserved when the flavin is bound.

Photoreactivation action spectra vary with the enzyme source, and maxima ranging from 360 to 445 nm have been reported. It has been suggested that these differences reflect structurally diverse chromophores bound to photolyase. In the case of the *E. coli* enzyme, the action spectrum can be accounted for by new near-ultraviolet absorbance between 300 and 400 nm of the enzyme–substrate complex (Wun et al., 1977); hence, it is not necessary to invoke the presence of a fluorescent chromophore to account for enzymatic activity. However, the photolyase from *Streptomyceus griseus* (Eker, 1978) is associated with a visible chromophore exhibiting an absorption maximum at 445 nm and a shoulder at 425 nm that coincide reasonably well with the action spectrum of the enzyme. While these data leave little doubt that a chromophore is bound to the *S. griseus* enzyme, they are inadequate to delineate it as a reduced flavin.

Efforts to detach the chromophore without denaturing yeast photolyase have not proved fruitful; hence, whether it is involved in photoreactivation is still not resolved. The participation of the chromophore of the yeast enzyme has been questioned because action spectra differ for irradiated dG:dC and dA:dT (Rupert & To, 1976). However, the marked dependence of the spectral properties of reduced flavins upon their immediate environment could account for this difference (Ghisla et al., 1974).

It has been reported that an adenine-containing moiety bound to *E. coli* photolyase contributes to the 257-nm absorption of the native enzyme (Snapka & Fuselier, 1977). There is a reversible loss of activity upon dissociation of the moiety from the enzyme, and absence of near-UV absorption precludes its identification as a flavin.

The photolyase reported by Boatwright et al. (1975) differs from the one described here and by Minato & Werbin (1971) in the following respects: it has an average molecular weight of 133 000 and two dissimilar subunits. These distinctions and a recent report by Madden (1979) raise the possibility that there are two DNA photolyases in various batches of commercial yeast.

Acknowledgments

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Stereochemical and Kinetic Studies on the Action of the Catalytic Subunit of Bovine Cardiac Muscle Adenosine 3′,5′-Monophosphate Dependent Protein Kinase Using Metal Ion Complexes of ATPβS†

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ABSTRACT: The phosphotransferase activity of bovine cardiac muscle protein kinase catalytic subunit has been investigated by the use of metal ion complexes of ATPβS diastereomers ("A" and "B") as donor substrates in peptide phosphorylation. It was found that MgATPβS "A" was used by the enzyme 500-fold faster than MgATPβS "B" but that Cd^{II}ATPβS "B" was preferred by about 20-fold over Cd^{II}ATPβS "A" isomer. It is argued that the β,γ-bidentate complexes of MgATPβS "A" and of Cd^{II}ATPβS "B", as well as the Δ isomer of $Co^{III}(NH_3)_4$ATP, have identical stereochemistry about the β-phosphorus position and that this structure is preferentially recognized by the enzyme. A comparison of double-reciprocal kinetic plots suggested that the reaction with MgATPβS "A" proceeds by a ping-pong mechanism while that with MgATP follows an ordered sequence. Further analysis including product inhibition and γ-^{32}P exchange studies showed that a ping-pong mechanism is not plausible for MgATPβS "A" and that, like MgATP, phosphorylation employing the "A" isomer probably proceeds by an ordered sequence. The enzyme uses MgATPβS "A" and MgATP as substrates in very much the same manner since the same k_{cat} (~640 min^{-1}) and K_m (10.7 μM) values are obtained with either substrate. Kinetic differences between the reactions of the two nucleotide triphosphate substrates arise from different K_m values for peptide and, presumably, from different dissociation constants for the enzyme–Mg–nucleotide triphosphate complexes.

The stereochemistry of the metal ion–nucleotide triphosphate complexes accepted as substrates by pyruvate kinase and yeast hexokinase has recently been examined (Jaffe & Cohn, 1978) through the use of the "A" and "B" diastereomers of ATPβS (Eckstein & Goody, 1976).[1] Cornelius & Cleland (1978) showed that hexokinase reacts with the Λ diastereomer of the stable metal ion β,γ-bidentate complex $Co^{III}(NH_3)_4$ATP, and the absolute configuration of the Co(III)–triphosphate moiety has been established (Merritt et al., 1978). The center of asymmetry of $Co^{III}(NH_3)_4$ATP and MgATPβS in the triphosphate chain is the β-phosphate position, and for each β,γ-bidentate complex of $Co^{III}(NH_3)_4$ATP there exists a MgATPβS diastereomer of the same geometric configuration. On the basis of the stereoselectivity of hexokinase for the MgATPβS "B" diastereomer, Jaffe & Cohn (1978) assigned this isomer as having the same stereochemistry as the Λ isomer of $Co^{III}(NH_3)_4$ATP.

To elucidate the stereochemistry of the metal ion–nucleotide triphosphate complex which reacts with the catalytic subunit of bovine cardiac muscle cAMP-dependent protein kinase (EC 2.7.1.37; ATP:protein phosphotransferase), we have investigated the action of the catalytic subunit on metal ion complexes of ATPβS. Granot et al. (1979) have shown that the Δ isomer of β,γ-bidentate $Co^{III}(NH_3)_4$ATP reacts with the protein kinase catalytic subunit, albeit very slowly, while the Λ isomer does not appear to serve as a substrate. Though there is a definite preference exhibited by a number of enzymes for reaction with either the Λ or Δ isomer of $Co^{III}(NH_3)_4$ATP, the rate of utilization of the preferred Co(III) complex is invariably quite slow (Danenberg & Cleland, 1975; Cornelius & Cleland, 1978; Li et al., 1978; Dunaway-Mariano et al., 1979). Because of the low rate of reaction, we found it desirable to employ additional stereochemical probes involving

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[1] Abbreviations used: we have used the same designation ("A" and "B") for the diastereomers of ATPβS as employed by Eckstein & Goody (1976); PEI, poly(ethylenimine); DTT, dithiothreitol.

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