Evidence for the Existence of a Tyrosyl Residue in the Nicotinamide Adenine Dinucleotide Binding Site of Chicken Liver Xanthine Dehydrogenase†

Tomoko Nishino and Takeshi Nishino*

Department of Biochemistry, Yokohama City University School of Medicine, Fukuura 3-9, Yokohama 236, Japan

Received October 15, 1986; Revised Manuscript Received January 6, 1987

ABSTRACT: Xanthine–NAD and NADH–methylene blue oxidoreductase activities of chicken liver xanthine dehydrogenase were inactivated by incubation with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), an active site directed reagent for nucleotide binding sites. The inactivation reaction displayed pseudo-first-order kinetics. A double-reciprocal plot of inactivation velocity vs. 5′-FSBA concentration showed that 5′-FSBA and enzyme formed a complex prior to inactivation. NAD protected the enzyme from inactivation by 5′-FSBA in a competitive fashion. The modified enzyme had the same xanthine–dichlorophenolindophenol and xanthine–O_2 oxidoreductase activities as the native enzyme, and on addition of xanthine to the modified enzyme, bleaching of the spectrum occurred in the visible region. The amount of radioactivity incorporated into the enzyme by incubation with [^{14}C]-5′-FSBA was parallel to the loss of xanthine–NAD oxidoreductase activity, and the stoichiometry was 1 mol/mol of enzyme-bound FAD for complete inactivation. These results indicated that 5′-FSBA modified specifically the binding site for NAD of chicken liver xanthine dehydrogenase. The incorporated radioactivity was released slowly from ^{14}C-labeled enzyme by incubation with dithiothreitol with concomitant restoration of catalytic activity. The modified residue responsible for inactivation was identified as a tyrosine.

Chicken liver xanthine dehydrogenase (xanthine–NAD oxidoreductase, EC 1.2.1.37) is an enzyme very similar to milk xanthine oxidase (Bray, 1975, 1981). They have similar molecular weights and have wide substrate specificities. They contain one FAD, one molybdenum, and two Fe_2S_2 iron–sulfur centers per subunit as prosthetic groups. Mammalian xanthine oxidases including the milk enzyme are known to exist mostly as NAD-dependent types in freshly prepared samples (Della Corte & Stirpe, 1968, 1972; Stirpe & Della Corte, 1969; Battelli et al., 1972; Waud & Rajagopalan, 1976a; Nakamura & Yamazaki, 1982), and the NAD-dependent type was known to be convertible to the O_2-dependent type reversibly by thiol oxidation and irreversibly by proteolysis (Della Corte & Stirpe, 1972; Waud & Rajagopalan, 1976b). However, in contrast to mammalian enzymes, the chicken liver enzyme has never been shown to be converted to the O_2-dependent type. Despite the study of these interconversion processes, the chemical nature of the group(s) that determines (determine) the properties of FAD in the different enzyme forms remains obscure. Structural comparison between avian and mammalian enzymes would seem to be important for understanding the mechanism of interconversion between the two forms.

Although there is a vast literature on xanthine oxidase and xanthine dehydrogenase, most studies have concentrated on the behavior of the various oxidation–reduction groups of the enzyme during catalysis; little literature is available on protein structure and chemical modification (Massey, 1973; Bray, 1975, 1981). It is only known that a lysyl residue exists near the molybdenum in milk xanthine oxidase (Nishino et al., 1982) and a sulfhydryl group (or groups) exists near the FAD or NAD binding site and is involved in the interconversion between NAD-dependent and O_2-dependent types of rat liver xanthine dehydrogenase (Battelli et al., 1973; Waud & Rajagopalan, 1976b).

In order to obtain information about the structure of the NAD binding site of chicken liver enzyme, we tried to modify specifically the NAD binding site with the active site directed reagent of 5′-FSBA,[1] a NAD analogue (Colman et al., 1977).

Materials and Methods

Xanthine dehydrogenase was purified from livers of chickens fed a high-protein diet by the procedure of Nishino (1974). Xanthine, 5′-FSBA, *N*-acetyltyrosine, and *N*-acetyllysine were purchased from Sigma, NAD and NADH from Oriental Yeast Co., and methylene blue and DCI from Wako Chemicals. *p*-(Fluorosulfonyl)benzoic acid was purchased from Aldrich, and dithiothreitol was from Boehringer Mannheim. CBS-tyrosine and CBS-lysine were synthesized by the procedure of Likos et al. (1980). All other reagents were of analytical grade. [*adenosyl*-^{14}C]-5′-FSBA was obtained from New England Nuclear and was diluted with cold 5′-FSBA to a specific activity of 12 140 dpm/nmol. Specific activity was checked by measuring the concentration of 5′-FSBA with a molar extinction of $1.58 \times 10^4 M^{-1}$ cm^{-1} at 259 nm (Colman et al., 1977). Xanthine dehydrogenase activities were determined at 25 °C in 0.05 M potassium phosphate buffer, pH 7.8, containing 0.4 mM EDTA. The oxidation of xanthine (150 μM) by the various acceptors and the wavelengths at which reactions were monitored were as follows: NAD (500 μM), 340 nm; DCI (50 μM), 600 nm; and methylene blue (50 μM), 295 nm. The oxidations of NADH (50 μM) by methylene blue and DCI were followed at 340 and 600 nm, respectively. The enzyme concentration was determined spec-

†This work was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture (58480142) and in part by a research grant for the intractable diseases from the Japanese Ministry of Health and Welfare.

* Author to whom correspondence should be addressed.

[1] Abbreviations: 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; DCI, dichlorophenolindophenol; CBS-tyrosine, *O*-(4-carboxybenzenesulfonyl)tyrosine; CBS-lysine, N^{ϵ}-(4-carboxybenzenesulfonyl)lysine; CBS-amino acid, (carboxybenzenesulfonyl)-amino acid; 5′-SBA, 5′-(*p*-sulfonylbenzoyl)adenosine; TLC, thin-layer chromatography.

0006-2960/87/0426-3068$01.50/0

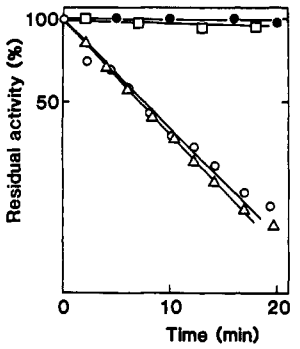

FIGURE 1: Semilogarithmic plot of time course of inactivation of chicken liver xanthine dehydrogenase by 5′-FSBA. At 25 °C, 2.5 μM enzyme was incubated in 50 mM phosphate buffer, pH 7.8, with 127 μM 5′-FSBA. Aliquots were withdrawn at the indicated times for determination of activities of xanthine–NAD (○), NADH–DCI (△), xanthine–DCI (●), and xanthine–O_2 (□) oxidoreductases as described under Materials and Methods.

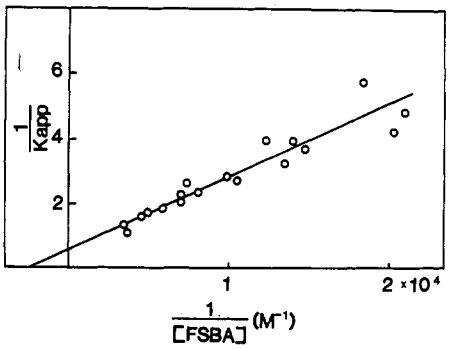

FIGURE 2: Double-reciprocal plot of apparent rate constant of inactivation (k_{app}) vs. 5′-FSBA concentration. Enzyme (2 μM) was incubated with various concentrations of 5′-FSBA under the same conditions as described in Figure 1. Apparent pseudo-first-order rate constants were obtained from plots of log (percent residual xanthine–NAD oxidoreductase activity) vs. time. The values of K_i for 5′-FSBA and of V_{max} were determined to be 0.4 mM and 2.2 min^{-1}, respectively.

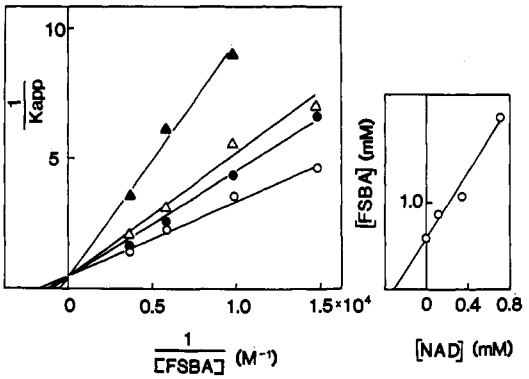

FIGURE 3: Effect of NAD on inactivation of enzyme with 5′-FSBA. (Left) Double-reciprocal plots of apparent rate constant (k_{app}) vs. 5′-FSBA concentration at a series of a fixed concentration of NAD (○, no addition of NAD; ●, 120 μM NAD, △, 360 μM NAD; ▲, 723 μM NAD). Enzyme (1.7 μM) was incubated, and apparent rate constants were obtained as described in Figure 2. (Right) Secondary plot of left-side figure. K_d for NAD was determined to be 3.2×10^{-4} M.

trophotometrically with a value of 37 000 M^{-1} cm^{-1} for the molar absorbance at 450 nm (Rajagopalan & Handler, 1967). Absorption spectra were recorded with an Aminco-Chance DW-2a spectrophotometer or a Hitachi 557 spectrophotometer. Modification of the enzyme with 5′-FSBA was performed in 0.05 M potassium phosphate buffer, pH 7.8, at 25 °C with various concentrations of 5′-FSBA. Various concentrations of 5′-FSBA, which were previously dissolved in dimethylformamide, were added to the enzyme solution. No effect of dimethylformamide (final concentration <5%) on the enzyme activity was confirmed. Experiments with [^{14}C]-5′-FSBA were performed in the same buffer at 0 °C. After various incubation periods, aliquots were withdrawn, and residual catalytic activity and radioactivity were determined. For determination of radioactivity, aliquots were mixed with chilled ethanol to 50%, and the resulting precipitates were collected on membrane filters (Sartorius) at 0–4 °C. The precipitate on the membrane filter was washed through with ice-cold 50% ethanol, and after air-drying, radioactivity was counted in Aquasol 2 from New England Nuclear with a Packard liquid scintillation counter.

CBS-amino acids were identified essentially by the procedure of Saradambal et al. (1981). The enzyme modified with 5′-FSBA was hydrolyzed for 20 h in 6 N HCl at 110 °C. Hyrolysate or standard CBS-amino acids were spotted on cellulose thin-layer chromatography plates (a type without fluorescence obtained from Eastman Kodak Co.) and subjected first to electrophoresis in the horizontal direction at pH 6.3 in pyridine/acetic acid/water (25:1:250) at 300 V/20 cm for 90 min. After the plates were dried, they were developed in the vertical direction by butanol/acetic acid/water (4:1:5). Amino acids and CBS-amino acids were detected by the ninhydrin reaction.

RESULTS

Inactivation of Chicken Liver Xanthine Dehydrogenase by 5′-FSBA. 5′-FSBA was found to inactivate NAD-dependent activities of chicken liver xanthine dehydrogenase. As shown in Figure 1, xanthine–NAD and NADH–DCI oxidoreductase activities of xanthine dehydrogenase were inactivated time dependently by incubation with 5′-FSBA, whereas xanthine–DCI and xanthine–O_2 oxidoreductase activities were maintained at the initial level. These results suggested that 5′-FSBA attacked not the xanthine but the NAD binding site. The inactivation of xanthine–NAD oxidoreductase activity displayed pseudo-first-order kinetics with various concentrations of 5′-FSBA. The double-reciprocal plots of apparent rate constant (k_{app}) vs. 5′-FSBA concentration showed that 5′-FSBA and enzyme formed a reversible complex prior to the inactivation reaction (Figure 2). This type of kinetics is described by

$$E + 5'\text{-FSBA} \rightleftharpoons E\text{–}5'\text{-FSBA} \rightarrow E' \qquad (1)$$

where E–5′-FSBA is a reversible complex of enzyme and 5′-FSBA and E′ is the inactivated form of the enzyme. The observed rate constant for inactivation (k_{obsd}) is expressed as

$$k_{obsd} = k/(1 + K_i/[5'\text{-FSBA}]) \qquad (2)$$

where k_{obsd} is the apparent rate constant at a particular concentration of 5′-FSBA, K_i is the apparent dissociation constant of the enzyme–5′-FSBA complex, and k is the intrinsic rate constant for covalent modification of the enzyme observed at infinitely high concentrations of the reagent. The fit of the data of Figure 2 to the reciprocal form of the equation yielded a value of $K_i = 4 \times 10^{-4}$ M for the enzyme–reagent complex with $k = 2.2$ min^{-1}.

Effect of NAD on Reaction Rate of Enzyme with FSBA. That modification of the enzyme with 5′-FSBA was near the NAD binding site was supported by the fact that inactivation of the enzyme was strongly protected by NAD. If 5′-FSBA is an active site directed reagent, it should compete with NAD for the same binding site. As shown in Figure 3 the double-reciprocal plots of inactivation rate vs. 5′-FSBA concentration

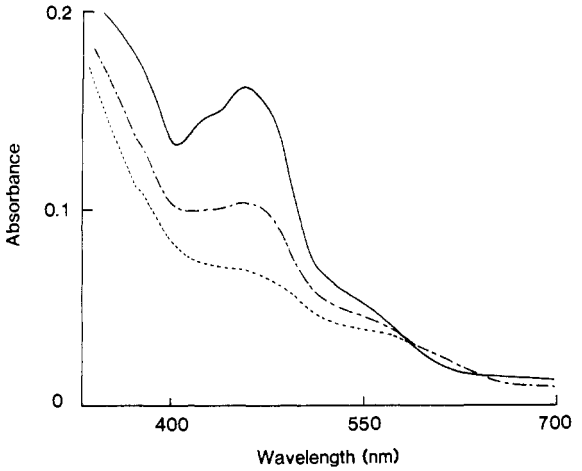

FIGURE 4: Spectral change of 5′-FSBA-modified enzyme with xanthine. Chicken liver xanthine dehydrogenase modified with 5′-FSBA (residual activities of xanthine–NAD and xanthine–DCI oxidoreductases were 5.6% and 92.6%, respectively) was mixed with 1.92 mM xanthine under anaerobic condition at 25 °C, and absorption spectra were taken before (—), immediately after (- - -), and 24 h after (- - -) the enzyme was mixed with xanthine.

with different NAD concentrations shows that the kinetic pattern of inactivation by 5′-FSBA is competitive with NAD. For such competition, the observed rate of inactivation is expressed by

$$k_{obsd} = k/[1 + (1 + [NAD]/K_d)K_i/[5'\text{-FSBA}]] \quad (3)$$

where K_i and k are defined as above and K_d is the dissociation constant of the enzyme–NAD complex. From secondary plots of apparent K_i vs. NAD concentration the K_d for NAD was obtained to be 3.2×10^{-4} M. This value is much larger than the K_m value for NAD obtained from steady-state kinetic analyses by Rajagopalan and Handler (1967). The lower affinity of enzyme for NAD obtained in the present experiment might be explained by the mechanism of reaction in this enzyme. Analyses of steady-state kinetics (Rajagopalan & Handler, 1967) revealed that the reaction of xanthine–NAD oxidoreductase followed the ping-pong mechanism, where NAD bound strongly to reduced enzyme. In the present case the K_d measured is that for binding of NAD to oxidized enzyme.

Absorption Spectra of Modified Enzyme. When the 5′-FSBA-inactivated enzyme was mixed with xanthine under anaerobic conditions, considerable bleaching of the visible spectrum was observed (Figure 4), indicating that the redox chromophores of the modified enzyme were able to be reduced by xanthine and that reoxidation of flavin by NAD might be interrupted by modification of the enzyme by 5′-FSBA. This agreed with the finding that only NAD-dependent activities were lost by modification.

Incorporation of Radioactivities from [adenosyl-^{14}C]-5′-FSBA into the Enzyme. The stoichiometry of the reaction was determined by measurement of the incorporation of [^{14}C]-5′-FSBA labeled at the C-8 position of adenosine. Figure 5 shows a linear correlation between the percentage activity and [*adenosyl*-^{14}C]-5′-SBA residue incorporated into the enzyme. By extrapolation, a value of 1 mol of 5′-SBA residue/mol of enzyme–FAD was obtained for fully inactivated enzyme. Incorporation of radioactivity was prevented when the enzyme was incubated with [^{14}C]-5′-FSBA in the presence of NAD even under severe conditions. Only 0.14 mol of [^{14}C]-5′-SBA was incorporated after incubation of the enzyme with 0.1 mM [^{14}C]-5′-FSBA in the presence of 10 mM NAD at 25 °C for 40 min.

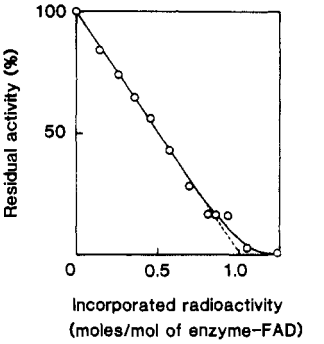

FIGURE 5: Stoichiometry of incorporation of [^{14}C]-5′-SBA residue into chicken liver xanthine dehydrogenase. At 0 °C, 5.8 μM enzyme was incubated with 78 μM of [^{14}C]-5′-FSBA in 50 mM potassium phosphate buffer, pH 7.8, containing 0.2 mM EDTA. After incubation for various periods, residual activity and radioactivity were determined as described under Materials and Methods.

Reactivation of 5′-FSBA-Inactivated Enzyme by Dithiothreitol. The 5′-FSBA-inactivated enzyme could be reactivated very slowly by incubation with relatively high concentrations of dithiothreitol (20 mM) at 37 °C. Enzyme having 10% of its original activity could be reactivated to about 60% of its original NADH–methylene blue activity after 4 h and to more than 80% after 8 h. However, only 50% of the original xanthine–NAD oxidoreductase activity could be reactivated after 8 h by this incubation. Under the reactivation condition, xanthine–methylene blue and xanthine–NAD activities of native enzyme were inactivated partially (about 80% of its original activity after 8 h) whereas NADH–methylene blue activity was not affected. Limited reactivation of xanthine–NAD oxidoreductase activity might be partially due to inactivation during incubation with dithiothreitol, because prolonged incubation with dithiothreitol was found to release the essential sulfur atom at molybdenum (T. Nishino, unpublished observation).

The amount of release of [^{14}C]-5′-SBA was estimated by determination of remaining radioactivity in the enzyme before and after incubation of the [^{14}C]-5′-FSBA-inactivated enzyme with dithiothreitol. By incubation of [^{14}C]-5′-FSBA-inactivated enzyme (remaining xanthine–NAD oxidoreductase activity, 18% of its original activity) with dithiothreitol at 37 °C overnight, 0.58 mol of radioactivity/mol of enzyme–FAD was released with activity increase of 47%. Under the incubation conditions, 13% of incorporated radioactivity was released from the [^{14}C]-5′-FSBA-inactivated enzyme by incubation without dithiothreitol. This release was not accompanied by any reactivation of catalytic activity and therefore is likely to be due to hydrolysis of the ester linkage between the benzoyl and adenosyl moieties. After correction of the release without reactivation, the amount of radioactivity released by incubation with dithiothreitol was found to be almost stoichiometric with the percentage of reactivation. The radioactive compound released by incubation of the ^{14}C-labeled enzyme both with and without dithiothreitol was identified to be [^{14}C]adenosine on TLC. It was considered that the [^{14}C]adenosine might be derived from free [^{14}C]-5′-SBA, which was released from the ^{14}C-labeled enzyme, by hydrolysis of the ester linkage between the benzoyl and adenosine moieties during incubation, since free 5′-FSBA was found to hydrolyze to adenosine and carboxybenzenesulfonate by incubation under the reactivation conditions. The experiment of further remodification of the reactivated enzyme with [^{14}C]-5′-FSBA showed that a stoichiometric amount of radioactivity was again incorporated into the enzyme with concomitant loss of activity (data not shown), indicating that the reactive residue was regenerated by incu-

bation of modified enzyme with dithiothreitol and supporting the conclusion that 5′-SBA was released by incubation with dithiothreitol with concomitant restoration of catalytic activity.

Identification of the Amino Acid Residue Modified during Inactivation by 5′-FSBA. An acid hydrolysate of xanthine dehydrogenase, which had been inactivated to an extent of 40% (where minimum nonspecific incorporation of CBS residue was expected), was applied to two-dimensional thin-layer chromatography as described under Materials and Methods. Under the condition of hydrolysis the ester linkage between the benzoyl and adenosyl moieties are hydrolyzed to yield the corresponding CBS-amino acids (Saradambal et al., 1981). Under the condition of performed TLC, only glutamate, aspartate, CBS-lysine, and CBS-tyrosine are negatively charged, and the latter two compounds are rather hydrophobic (Saradambal et al., 1981). Thus, in comparison with standard CBS-tyrosine and CBS-lysine, the modified amino acid in the acid hydrolysate of 5′-FSBA-modified enzyme was readily identified to be a CBS-tyrosine.

Discussion

5′-FSBA exhibits many of the characteristics of an active site directed reagent for the NAD binding site of chicken liver xanthine dehydrogenase. Kinetics of inactivation showed that the enzyme and 5′-FSBA formed a complex prior to inactivation. Protection of inactivation by NAD was competitive with 5′-FSBA, indicating that the site of modification by 5′-FSBA was the NAD binding site. This was consistent with the result that only NAD- or NADH-dependent activities were inactivated, while xanthine–DCI or xanthine–O_2 oxidoreductase activities were not affected by the modification. This was also consistent with the spectral observation that the modified enzyme could be reduced with xanthine.

The incorporation of radioactive [^{14}C]-5′-SBA was parallel with the loss of xanthine–NAD oxidoreductase activity with a stoichiometry of 1 mol/mol of enzyme-bound FAD for complete inactivation. The incorporation of radioactivity was prevented in the presence of NAD. By incubation of [^{14}C]-5′-FSBA-modified enzyme with dithiothreitol, the incorporated radioactivity was released with concomitant restoration of catalytic activity. The reactivated enzyme could be again modified with 5′-FSBA. These results indicate that incorporation of 5′-SBA into the enzyme has caused inactivation of catalytic activity. Conversion of sulfhydryl residues into a disulfide bond during modification, of which examples have been reported in modification of some enzymes with 5′-FSBA (Likos & Coleman, 1981; Tomichi et al., 1981; Togashi & Reisler, 1982; Takata & Fujioka, 1984), is not likely to be the case. This is consistent with the finding that no inactivation occurs by incubation of the enzyme with thiol reagents such as *p*-(chloromercuri)benzoic acid (PCMB), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), dithiodipyridine, and iodoacetic acid (data not shown). Titrations of sulfhydryl residues in the presence of guanidine hydrochloride have not been performed because quite a large number of sulfhydryl residues are known to exist in this enzyme (T. Nishino, unpublished result). The fact that the reactivation rate of 5′-FSBA-modified enzyme by dithiothreitol is much slower than that of reported examples of disulfide reduction also supports this conclusion.

The residue reacted with 5′-FSBA was identified as a tyrosine after acid hydrolysis of the modified enzyme. This is consistent with the property of susceptibility of the 5′-SBA–enzyme to dithiothreitol. The slow reactivation of the 5′-SBA–enzyme by dithiothreitol suggested that the modified residue must be susceptible to nucleophilic replacement. A tyrosyl residue as well as cysteinyl and histidyl residues would fulfill this property. On incubation of free benzoyltyrosine with dithiothreitol under the same condition as reactivation, free tyrosine was found to be formed by analysis on TLC. The existence of a tyrosyl residue in an NAD binding site is not very common. However, modification of a tyrosyl residue by 5′-FSBA in nucleotide binding sites of some enzymes has been reported (Esch & Allison, 1978; Likos et al., 1980; Saradambal et al., 1981).

Mammalian xanthine dehydrogenase of the NAD-dependent type is convertible to the O_2-dependent type with modification of cysteinyl residues which were suspected to exist near the FAD or NAD binding site (Battelli et al., 1973). However, in this study it was found that blocking of the NAD binding site did not change the reactivity for oxygen of chicken liver xanthine dehydrogenase. It has been pointed out that the flavin redox potential and the stability of the semiquinone are significantly different in these two forms (Waud & Rajagopalan, 1976; Bray et al., 1976; Barber et al., 1977). From the present results it is likely that blocking of the NAD binding site is not important for the interconversion.

Acknowledgments

We express our gratitude to Dr. Keizo Tsushima, Yokohama City University, and Dr. Vincent Massey, The University of Michigan, for helpful discussions and critical reading of the manuscript.

References

Barber, M. J., Bray, R. C., Commack, R., & Coughlan, M. P. (1977) *Biochem. J. 163*, 279–289.

Battelli, M. G., Della Corte, E., & Stirpe, F. (1972) *Biochem. J. 126*, 747–749.

Battelli, M. G., Lorenzoni, E., & Stirpe, F. (1973) *Biochem. J. 131*, 191–198.

Bray, R. C. (1975) *Enzymes (3rd Ed.) 12*, 299–419.

Bray, R. C. (1981) in *Flavins and Flavoproteins* (Massey, V., & Williams, C. H., Jr., Ed.) pp 775–785, Elsevier/North-Holland, New York.

Colman, R. F., Pal, P. K., & Wyatt, J. L. (1977) *Methods Enzymol. 66*, 240–249.

Della Corte, E., & Stirpe, F. (1968) *Biochem. J. 108*, 349.

Della Corte, E., & Stirpe, F. (1972) *Biochem. J. 126*, 739–745.

Esch, F. S., & Allison, W. S. (1978) *J. Biol. Chem. 253*, 6100–6106.

Likos, J. J., & Colman, R. F. (1981) *Biochemistry 20*, 491–499.

Likos, J. J., Hess, B., & Colman, R. F. (1980) *J. Biol. Chem. 255*, 9388–9398.

Massey, V. (1973) in *Iron–Sulfur Proteins* (Lovenberg, W., Ed.) Vol. 1, pp 301–360, Academic, New York.

Nakamura, M., & Yamazaki, I. (1982) *J. Biochem. (Tokyo) 92*, 1279–1286.

Nishino, T. (1974) *Biochim. Biophys. Acta 341*, 93–98.

Nishino, T., Tsushima, K., Hille, R., & Massey, V. (1982) *J. Biol. Chem. 257*, 7358–7363.

Prasad, A. R. S., Ybarra, J., & Nishimura, J. S. (1983) *Biochem. J. 215*, 513–518.

Rajagopalan, K. V., & Handler, P. (1967) *J. Biol. Chem. 242*, 4097–4107.

Saradambal, K. V., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 11866–11872.

Stirpe, F., & Della Corte, E. (1969) *J. Biol. Chem. 244*, 3855–3863.

Takata, Y., & Fujioka, M. (1984) *Biochemistry 23*, 4357–4362.

Togashi, C. T., & Reisler, E. (1982) *J. Biol. Chem. 257*, 10112–10118.
Tomichi, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.
Waud, W. R., & Rajagopalan, K. V. (1976a) *Arch. Biochem. Biophys. 172*, 354–364.
Waud, W. R., & Rajagopalan, K. V. (1976b) *Arch. Biochem. Biophys. 172*, 365–379.

A Probe for the Mutagenic Activity of the Carcinogen 4-Aminobiphenyl: Synthesis and Characterization of an M13mp10 Genome Containing the Major Carcinogen–DNA Adduct at a Unique Site†

Dana D. Lasko,‡,§ Ashis K. Basu,‡ Fred F. Kadlubar,∥ Frederick E. Evans,∥ Jackson O. Lay, Jr.,∥ and John M. Essigmann*,‡

Department of Applied Biological Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, and Division of Biochemical Toxicology, National Center for Toxicological Research, Jefferson, Arkansas 72079

Received October 7, 1986; Revised Manuscript Received January 5, 1987

ABSTRACT: The duplex genome of *Escherichia coli* virus M13mp10 was modified at a unique site to contain *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl ($dG^{8\text{-}ABP}$), the major carcinogen–DNA adduct of the human bladder carcinogen 4-aminobiphenyl. A tetradeoxynucleotide containing a single $dG^{8\text{-}ABP}$ residue was synthesized by reacting 5′-d(TpGpCpA)-3′ with *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl, followed by high-performance liquid chromatography purification of the principal reaction product 5′-d(TpG$^{8\text{-}ABP}$pCpA)-3′ (yield 15–30%). Characterization by fast atom bombardment mass spectrometry confirmed the structure as an intact 4-aminobiphenyl-modified tetranucleotide, while ^1H nuclear magnetic resonance spectroscopy established the site of substitution and the existence of ring stacking between the carcinogen residue and DNA bases. Both 5′-d(TpG$^{8\text{-}ABP}$pCpA)-3′ and 5′-d(TpGpCpA)-3′ were 5′-phosphorylated by use of bacteriophage T4 polynucleotide kinase and were incorporated into a four-base gap uniquely positioned in the center of the recognition site for the restriction endonuclease *Pst*I, in an otherwise duplex genome of M13mp10. In the case of the adducted tetranucleotide, $dG^{8\text{-}ABP}$ was located in the minus strand at genome position 6270. Experiments in which the tetranucleotides were 5′ end labeled with [^{32}P]phosphate revealed the following: (i) the adducted oligomer, when incubated in a 1000-fold molar excess in the presence of T4 DNA ligase and ATP, was found to be incorporated into the gapped DNA molecules with an efficiency of approximately 30%, as compared to the unadducted d(pTpGpCpA), which was incorporated with 60% ligation efficiency; (ii) radioactivity from the 5′ end of each tetranucleotide was physically mapped to a restriction fragment that contained the *Pst*I site and represented 0.2% of the genome; (iii) the presence of the lesion within the *Pst*I recognition site inhibited the ability of *Pst*I to cleave the genome at this site; (iv) in genomes in which ligation occurred, T4 DNA ligase was capable of covalently joining both modified and unmodified tetranucleotides to the gapped structures on both the 5′ and the 3′ ends with at least 90% efficiency. Evidence also is presented showing that the $dG^{8\text{-}ABP}$-modified tetranucleotide was stable to the conditions of the recombinant DNA techniques used to insert it into the viral genome. On the basis of these and other data, the $dG^{8\text{-}ABP}$-modified genome was judged to be a useful probe for investigation of site-specific mutagenesis in *E. coli*.

Chemical carcinogens, when activated by metabolism to electrophilic forms, react with nucleophilic sites in DNA to form an array of carcinogen–DNA adducts (Miller, 1978a). These products may be key intermediates in carcinogenesis, possibly by representing the distinct chemical lesions responsible for the mutagenic activation of oncogenes (Tabin et al., 1982; Sukumar et al., 1983). Indeed, the known mutational specificity of one carcinogen, *N*-methyl-*N*-nitrosourea, has been correlated with the specific DNA sequence change found following induction of rat mammary tumors after a single dose (Zarbl et al., 1985). In most cases, it has been exceedingly difficult to assign specific DNA adducts with responsibility for the mutational specificity of a chemical carcinogen, because so many different products form when carcinogens damage genomes. One simplifying approach to this problem is to situate a unique adduct in a phage or plasmid genome and then to replicate this defined substrate in cells to study site-directed mutagenesis in vivo. We have used this method in the past to show directly that O^6-methylguanine (O^6MeGua),[1] a minor lesion formed by methylating agents, causes G to A transition mutations in vivo (Loechler et al., 1984). This transition also is the genetic change observed in bacteria treated with alkylating agents that react with DNA by an S_N1 mechanism (Miller, 1978b) and, in the experiment cited above, in malignant tumors induced by *N*-methyl-*N*-nitrosourea.

The subject of this work is 4-aminobiphenyl (ABP), a human carcinogen found to produce bladder cancer in 17% of

† This investigation was supported by the National Institutes of Health (5 P01 ES00597, CA33821, and T32 ES07020).
* Address correspondence to this author.
‡ Massachusetts Institute of Technology.
§ Present address: Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts. EN6 3LD, U.K.
∥ National Center for Toxicological Research.

0006-2960/87/0426-3072$01.50/0