0026-895X/89/060818-05$02.00/0

MOLECULAR PHARMACOLOGY, 35:818-822

Reaction of Rat Liver DT-Diaphorase (NAD(P)H:Quinone Acceptor Reductase) with 5′-[*p*-(Fluorosulfonyl)benzoyl]-adenosine

XIAN-FANG LIU,[1] HENRY YUAN, MITSURU HANIU, TAKASHI IYANAGI, JOHN E. SHIVELY, and SHIUAN CHEN

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010 (X.-F.L., H.Y., M.H., J.E.S., S.C.) and Department of Biochemistry, University of Tsukuba, Niihari-gun, Ibaraki-ken, 305 Japan (T.I.)

Received January 26, 1989; Accepted March 28, 1989

SUMMARY

Rat liver DT-diaphorase is inactivated by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), following pseudo-first-order kinetics. A double-reciprocal plot of $1/k_{obs}$ versus 1/[5′-FSBA] yields a straight line with a positive *y* intercept, indicative of reversible binding of the inhibitor before an irreversible incorporation. The dissociation constant (K_d) for the initial reversible enzyme-inhibitor complex is estimated at 2.86 mM with $k_2 = 0.22$ min^{-1} (at pH 7.5 and 25°). A stoichiometry of 2 mol of 5′-FSBA bound/mol of enzyme (i.e., 1 mol of the inhibitor bound/mol of subunit), at 100% inactivation, was determined from inactivation kinetics and from incorporation studies using 5′-[*p*-(fluorosulfonyl)benzoyl]-[^{14}C]-adenosine. The irreversible inactivation as well as the covalent incorporation could be completely prevented by the presence of NAD(P)H during the incubation. These results indicate that 5′-FSBA inactivates DT-diaphorase by occupying its NAD(P)H binding site. Reverse phase high pressure liquid chromatography of tryptic digests of [^{14}C]5′-FSBA-labeled DT-diaphorase revealed one radioactive peak containing two comigrating peptides. They are ^{146}I-T-T-G-G-S-G-S-M-Y^{155} and ^{262}S-I-P-A-D-N-Q-I-K^{270}. By comparison of these sequences to those of the nucleotide binding sites of several kinases and dehydrogenases, it is suggested that the peptide I-T-T-G-G-S-G-S-M-Y is the one modified by 5′-FSBA and would be predicted to be the region where the pyrophosphate group of NAD(P)H binds.

DT-diaphorase [EC 1.6.99.2; NAD(P)H:quinone acceptor reductase] catalyzes a two-electron reduction of many types of quinones, including vitamin K_3 (menadione), to hydroquinones by either NADH or NADPH (1–3). This enzyme plays an important role in protecting tissues against the toxicity of quinones, which occur widely in nature, e.g., production of superoxide radicals and semiquinone free radicals (4–6). In addition, the liver enzyme is involved in the metabolism of vitamin K, which regulates the blood coagulation mechanism (7). Sequra-Aguilar *et al.* (8) presented data suggesting that the majority of NADPH-diaphorase activity measured in the brain can be attributed to DT-diaphorase. On the other hand, Kuonen *et al.* (9) suggested that the NADPH-dependent reduction of nitroblue tetrazolium HCl in brain tissue may be catalyzed by a different enzyme. Abnormally high NADPH-diaphorase activity in the brain has been implicated in the pathology of Huntington's disease (10). Neurons containing NADPH-diaphorase are selectively resistant to the endogenous toxic amino acids quinolinate and L-qlutamate. Selective sparing of neurons containing this enzyme is a hallmark of Huntington's disease (11), but the relationship between this enzyme and the disease has not been fully elucidated.

DT-diaphorase has unusual catalytic properties, in that it can utilize either NADH or NADPH as its coenzyme. Therefore, the active site of this enzyme can accommodate either NADH or NADPH. As a first step to determine the structure of the nicotinamide nucleotide binding site of this enzyme, we carried out an affinity labeling study using 5′-FSBA. This adenine analogue has been proven to be a useful affinity label for studies of many adenine nucleotide- and nicotinamide nucleotide-dependent enzymes (12), including our recent work on NADH-cytochrome b_5 reductase (13). Results presented here show that 5′-FSBA can be used to modify the nicotinamide nucleotide binding site of rat liver DT-diaphorase, resulting in inactivation of the enzyme. We have also attempted to characterize the 5′-FSBA-labeled peptide(s), and the results are discussed.

Experimental Procedures

Materials. 5′-FSBA was obtained from Sigma Chemical Co. (St. Louis, MO). 5′-Fluorosulfonylbenzoyl-[*adenine*-8-^{14}C]adenosine (36.2 mCi/mmol) was from New England Nuclear Corp. (Wilmington, DE).

This work was supported by National Institutes of Health Grants NS25786 and GM37297 (to S.C.) and GM34426 (to M.H.).

[1] Permanent address: Department of Clinical and Experimental Pharmacology, Dong Zhi-Men Hospital, Beijing College of Traditional Chinese Medicine, Beijing, People's Republic of China.

ABBREVIATIONS: 5′-FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; TPCK, tosylphenylalanine chloromethyl ketone; HPLC, high pressure liquid chromatography.

DT-diaphorase was purified from livers of female Wistar rats that were treated daily for 3 days with 3-methylcholanthrene (4 mg/100 g of body weight), using a procedure described by Haniu *et al.* (14).

Enzymatic assay. DT-diaphorase activity was determined spectrophotometrically by measuring the oxidation of NADH at 340 nm ($\epsilon_{340} = 6.22 \times 10^3$ M^{-1} cm^{-1}) at 25°. The assay mixture (1 ml) contained 50 mM sodium phosphate, pH 7.4, 197 μM NADH, and 160 μM menadione. The reaction was initiated with addition of the enzyme. The enzymatic assays were always performed in duplicate, and good agreement was always found between the two measurements. The specific activity of this enzyme preparation was 373 μmol of NADH oxidized/min/mg of protein.

Reaction of DT-diaphorase with 5′-FSBA. The reaction of DT-diaphorase with 5′-FSBA was carried out at 25° in 50 mM sodium phosphate buffer that contained 10% dimethylformamide, at pH 7.4. Inclusion of dimethylformamide at a level of 10% was required to maintain the solubility of 5′-FSBA over the incubation period but had no effect on the activity of DT-diaphorase. The extent of inactivation was monitored by measuring the residual enzyme activity at given time intervals with 10 μl of the 10-fold diluted incubation mixture.

Determination of the stoichiometry of 5′-FSBA labeling. DT-diaphorase (170 μg/ml) was incubated with [^{14}C]5′-FSBA (1.33 mCi/mmol) from 0 to 1.3 mM under the conditions given above. After 1 hr of incubation, the reaction mixture was immediately cooled to 4°. A 10-μl aliquot of each mixture was withdrawn for measurement of the residual enzyme activity. The rest of the sample was denatured in the presence of 1% SDS by incubation in a boiling water bath for 2 min. The labeled enzymes were then electrophoresed on SDS-polyacrylamide gel. The Coomassie blue-stained protein bands were sliced from the gel, and the gel pieces were dissolved in 0.1 ml of 30% hydrogen peroxide. The bound radioactivity was then evaluated. As a further confirmation of results, we also determined the amount of radioactivity associated with the enzyme by isolating the modified enzyme with reverse phase HPLC, using a Pierce RP-300 column (2 mm × 30 mm; 300 Å pore; 7 μm particle size). The modified proteins were well separated from the free analogues and were recovered in a good yield (above 90%).

Preparation and proteolytic digestion of 5′-FSBA-modified DT-diaphorase. DT-diaphorase (0.26 mg) was incubated with 3.6 μmol of [^{14}C]5′-FSBA (specific activity, 0.58 mCi/mmol) in a final volume of 2 ml. After a 1-hr incubation, the enzyme solution was dialyzed against water overnight, followed by a 4-hr dialysis against 100 mM ammonium bicarbonate, pH 8.0. The [^{14}C]5′-FSBA-modified protein was then digested with TPCK-trypsin in 100 mM ammonium bicarbonate for 24 hr at 37°. The ratio of TPCK-trypsin to 5′-FSBA-modified protein was approximately 1:50 (w/w).

Separation of peptides by reverse phase HPLC. The tryptic digest was subjected to reverse phase HPLC using an Altex Ultrasphere C18 column (4.6 mm × 250 mm; 5 μm particle size). The peptides were eluted with a linear 90-min gradient from 100% of solvent I (0.1% TFA) to 60% of solvent II (TFA/H_2O/CH_3CN, 0.1:9.9:90). Peptides were detected by absorbance at 220 nm and were collected manually, and 20 μl from each peak fraction were counted for radioactivity. Rechromatography of the radioactive peak was performed by reverse phase HPLC using an Ultremex 5 C18 column (4.6 mm × 250 mm) from Phenomenex, Inc. (Rancho Palos Verdes, CA), with a linear 60-min gradient from 95% of solvent I to 50% of solvent II.

Sequence analysis of the isolated peptide. Automated sequence analyses were performed on a gas phase sequencer, as described by Hawke *et al.* (15).

Results and Discussion

Inactivation of DT-diaphorase by 5′-FSBA. A time-dependent inactivation of rat liver DT-diaphorase was observed upon incubation with 5′-FSBA at 25° in 50 mM sodium phosphate buffer (pH 7.4) that contained 10% dimethylformamide. The initial inactivation followed pseudo-first-order kinetics, as indicated by the linear semilogarithmic plot of residual enzyme activity versus time of incubation (Fig. 1). The observed initial rate constants for inactivation (k_{obs}) at 0.435, 1.30, 1.74, 2.17, and 2.61 mM 5′-FSBA (these are concentrations during the incubation) were 0.029, 0.068, 0.077, 0.084, and 0.116 min^{-1}, respectively. Because the enzyme assay was performed with only 10 μl of the 10-fold diluted incubation mixture, the final concentrations of 5′-FSBA in the assay were 1/1000 of those during the incubation. 5′-FSBA concentrations up to 2.61 μM did not affect the DT-diaphorase activity when added directly during the enzyme assay.

The double-reciprocal plot of $1/k_{obs}$ versus 1/[5′-FSBA] yielded a straight line with a positive y intercept (Fig. 2), indicative of reversible binding of the inhibitor to the enzyme (E) before the irreversible incorporation (also see Ref. 16):

$$E + 5'\text{-FSBA} \overset{K_d}{\rightleftharpoons} E\text{- - -}5'\text{-FSBA} \overset{K_2}{\longrightarrow} E\text{-}5'\text{-FSBA} \quad (1)$$

From the y intercept of this double-reciprocal plot, we calculated the value of the first-order rate constant for inactivation, $K_2 = 0.22$ min^{-1}, and from the x intercept we calculated the dissociation constant for 5′-FSBA from the reversible complex

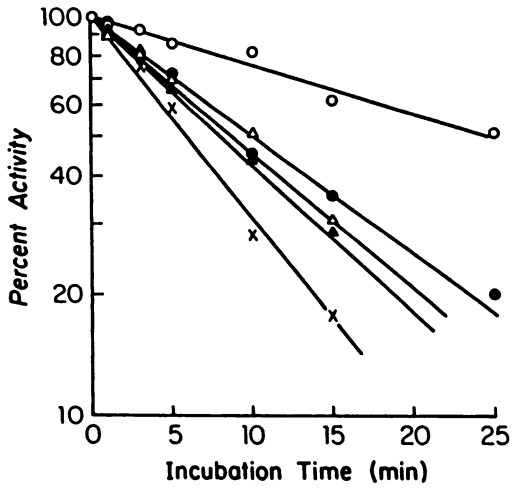

Fig. 1. Inactivation of DT-diaphorase by 5′-FSBA. Rat liver DT-diaphorase (170 μg/ml) was incubated with 0.435 (○), 1.30 (●), 1.74 (△), 2.17 (▲), or 2.61 mM (×) 5′-FSBA at 25° in 50 mM sodium phosphate buffer (pH 7.4) containing 10% dimethylformamide. At the times indicated, aliquots were withdrawn and assayed for enzyme activity.

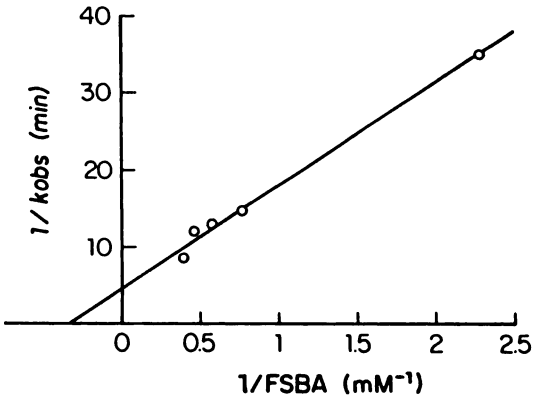

Fig. 2. Dependence of pseudo-first-order rate constants of inactivation reaction on 5′-FSBA concentration. The pseudo-first-order constants for the loss of enzyme activity, k_{obs} was calculated from curves as illustrated in Fig. 1.

(i.e., E– – –5′-FSBA), K_d = 2.86 mM. The control, which contained 10% dimethylformamide but not 5′-FSBA, maintained full activity under the same incubation conditions (data not shown).

Effect of nicotinamide nucleotide coenzymes on inactivation of DT-diaphorase by 5′-FSBA. NADPH, NADH, $NADP^+$ and NAD^+ all protected DT-diaphorase against 5′-FSBA inactivation in a concentration-dependent manner (Fig. 3). These results indicate that the 5′-FSBA modification occurs at the nicotinamide nucleotide binding site of DT-diaphorase. Presumably because of its lower affinity for the active site of the enzyme, NAD^+ is comparatively less effective in protection of DT-diaphorase from inactivation. We measured the enzyme activity with the diluted incubation mixture without the removal of nicotinamide nucleotides present in the mixture. As described above, the enzyme assay was performed with only 10 μl of the 10-fold diluted incubation mixture. Therefore, the final concentrations of protecting nucleotides were 1/1000 of those during the incubation. Because the amount of nicotinamide nucleotides present in the 10 μl of the diluted enzyme solution was much lower than that of the NADH we used as the substrate for enzyme assay (i.e., 197 nmol/ml), the nucleotides present in the diluted enzyme solution should not affect the enzyme activity. Indeed, we found that incubation of the enzyme with nicotinamide nucleotides up to 640 μM without 5′-FSBA did not affect the enzyme activity. This led us to conclude that reduction in the degree of 5′-FSBA inactivation of the enzyme by nicotinamide nucleotides was due to a competition between 5′-FSBA and protecting nucleotides for binding to the active site of the enzyme.

Stoichiometry of reaction of DT-diaphorase with 5′-FSBA. The stoichiometry of 5′-FSBA inactivation of DT-diaphorase was first determined by inactivation kinetics. Because the inactivation of DT-diaphorase by 5′-FSBA has been shown to proceed according to pseudo-first-order kinetics with respect to the concentration of 5′-FSBA, enzyme inactivation by this compound can be described by the following equation:

$$E + 5'\text{-FSBA} \xrightarrow{k} E\text{-}[5'\text{-FSBA}]^n \qquad (2)$$

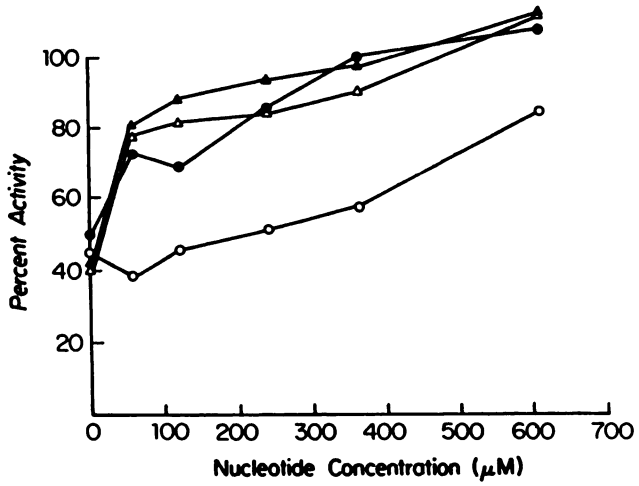

Fig. 3. Protection from 5′-FSBA inactivation of DT-diaphorase by pyridine nucleotides. The nucleotides used in this study are NADH (●), NAD^+, (○), NADPH (▲), and $NADP^+$ (△). DT-diaphorase (170 μg/ml) was incubated with 5′-FSBA at 1.5 mM and protecting nucleotides at the indicated concentrations. After a 15-min incubation at 25°, the enzyme activity was determined. The activity of a control without 5′-FSBA treatment was taken at 100%.

where n is the number of 5′-FSBA molecules bound per active site. Under our experimental conditions, where the concentration of 5′-FSBA is much greater than that of enzyme, the rate of enzyme inactivation is

$$\frac{d[E]}{dt} = k[E][5'\text{-FSBA}]^n \qquad (3)$$

and the pseudo-first-order rate constant (k_{obs}) is described by

$$k_{obs} = k\,[5'\text{-}FSBA]n \qquad (4)$$

and

$$\log k_{obs} = n \log [5'\text{-FSBA}] + \log k \qquad (5)$$

Eq. 5 can be used to determine the value of n, the inactivation stoichiometry. As illustrated in Fig. 4, the slope of the line obtained from a double logarithmic plot of K_{obs} versus [5′-FSBA] is 0.8, indicating that DT-diaphorase was inactivated by reaction with 1 mol of 5′-FSBA/mol of active site.

We also investigated the stoichiometry of the reaction of 5′-FSBA with DT-diaphorase by [^{14}C]5′-FSBA labeling. A plot of the enzyme inactivation versus moles of reagent incorporated per mole of enzyme revealed that 100% inactivation of the enzyme activity would be observed at 2 mol of 5′-FSBA bound/mol of enzyme (Fig. 5). Because this enzyme contains two identical subunits and one active site per subunit (17), these results confirm that DT-diaphorase was inactivated by reaction with 1 mol of 5′-FSBA/mol of active site or coenzyme binding site. Because the result obtained from labeling studies agrees very well with the results derived from inactivation kinetic analysis, we can conclude that 5′-FSBA binds very specifically only at the active site of the DT-diaphorase under our labeling conditions, resulting in inactivation of the enzyme. The nature of the slightly biphasic binding curve (Fig. 5) is not clearly understood, but it may suggest that a cooperative relationship exists between the two coenzyme binding sites in the two subunits. As described in Experimental Procedures, the [^{14}C]-5′-FSBA incorporation has been estimated by two different methods, determined after separation of the labeled enzyme from the unbound analogue either by SDS polyacrylamide gel electrophoresis or by reverse phase HPLC.

Attempted characterization of 5′-FSBA-labeled pep-

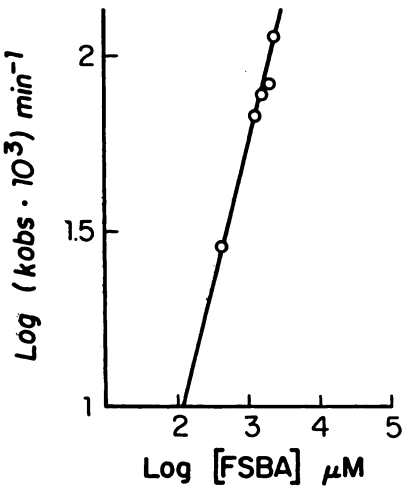

Fig. 4. Kinetic determination of the stoichiometry of the DT-diaphorase inactivation. The pseudo-first-order constant of inactivation of DT-diaphorase, k_{obs}, was obtained from Fig. 1.

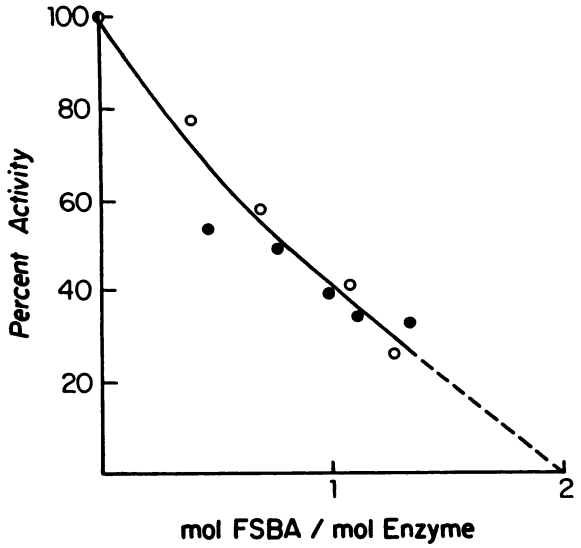

Fig. 5. Incorporation of 5′-FSBA into DT-diaphorase as a function of enzyme inactivation. DT-diaphorase (170 μg/ml) was incubated with [^{14}C]-5′-FSBA (1.33 mCi/mmol) from 0 to 1.3 mM for 60 min under the same conditions as those described in Fig. 1. After incubation, an aliquot of each sample was withdrawn for assay of enzyme activity. The labeled enzymes were isolated by either reverse phase HPLC (●) or SDS polyacrylamide gel electrophoresis (○) and counted for radioactivity as described in Experimental Procedures.

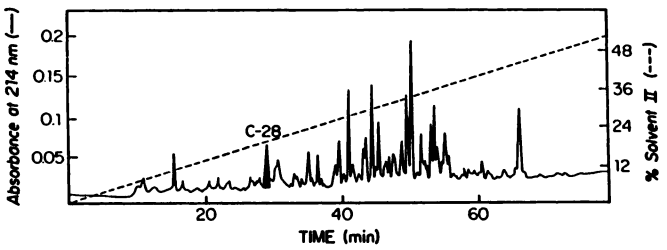

Fig. 6. Fractionation of [^{14}C]5′-FSBA-labeled peptides by reverse phase HPLC. The tryptic peptides of labeled enzyme were separated on an Altex Ultrasphere ODS C18 column (25 cm × 4.6 mm). A 90-min gradient program was run from 100% solvent I (0.1% TFA) to 60% solvent II (TFA/water/acetonitrile, 0.1:9.9:90, v/v/v) at a flow rate of 1 ml/min. Fractions were manually collected and a 20-μl aliquot from each peak fraction was counted for ^{14}C radioactivity.

TABLE 1

Sequence analysis of peptides in fraction C-28

Cycle	Peptide 1[a]		Peptide 2[b]	
	Amino acid released	Amino acid amount	Amino acid released	Amino acid amount
		pmol		*pmol*
1	Ile	43	Ser	3
2	Thr	13	Ile	16
3	Thr	16	Pro	13
4	Gly	24	Ala	19
5	Gly	35	Asp	8
6	Ser	3	Asn	11
7	Gly	17	Gln	4
8	Ser	3	Ile	—[c]
9	Met	2	Lys	—
10	Tyr	2		

[a] Approximately 50 pmol of peptide was subjected to sequence analysis.
[b] Approximately 25 pmol of peptide was subjected to sequence analysis.
[c] Not detected.

tides. In view of the very specific interaction of 5′-FSBA with DT-diaphorase, we decided to examine the site modified by this nucleotide probe in the enzyme. As described in Experimental Procedures, 0.26 mg of [^{14}C]5′-FSBA-labeled DT-diaphorase (corresponding to 70% inhibition of enzyme activity) was prepared. The stoichiometry of labeling was 1.24 mol of inhibitor incorporated/mol of enzyme. An NADH-protected enzyme, i.e., enzyme treated with [^{14}C]5′-FSBA in the presence of 5.3 mM NADH, was also prepared. These enzyme preparations were dialyzed against water overnight and digested with TPCK-trypsin in 100 mM ammonium bicarbonate. The tryptic fragments of the enzyme labeled in the absence of NADH, which were fractionated by reverse phase HPLC, yielded one major peak of ^{14}C radioactivity, C-28 (Fig. 6), whereas the NADH-protected preparation had full enzymatic activity and revealed no significant radioactive peak (results not shown), suggesting that this C-28 fraction of nonprotected sample contained the active site peptide(s) modified by 5′-FSBA. The radioactivity present in fraction C-28 was 48% of the original modified preparation, suggesting that approximately half of the radioactive derivative dissociated from the enzyme during trypsin digestion and the first HPLC separation. Rechromatography of fraction C-28 on an Ultremex 5 C18 column with a different solvent gradient resulted in two peaks, each of which contains one peptide. However, the radioactivity associated with fraction C-28 was lost following rechromatography, indicating that the labeling is not stable under such separation conditions, i.e., extensive incubation in acid. So far, we have not found separation conditions that would not release the labeling.

Although we have demonstrated kinetically that 5′-FSBA is a very specific active site-directed affinity label of DT-diaphorase, we were not able to definitively identify the 5′-FSBA-labeled peptide(s) because of the instability of the modified complex under analytical conditions. The amino acid sequences of two peptides in the radiolabeled fraction were determined. They are Ile-Thr-Thr-Gly-Gly-Ser-Gly-Ser-Met-Tyr and Ser-Ile-Pro-Ala-Asp-Asn-Glu-Ile-Lys (Table I). The amino acid sequence of rat liver DT-diaphorase has been deduced from the cDNA sequence (18, 19) and by direct protein sequencing (14). These two peptides are segments containing residues 146–155 and 262–270, respectively.

Because we did not find any additional sequences in the fraction C-28, we conclude that 5′-FSBA must have bound to at least one of these two peptides. Because of the instability of the labeling, we could not determine which of the two peptides contained the label or which amino acid residue(s) was involved in binding. The possibility that the radioactivity associated with this HPLC fraction was due to comigration of unbound [^{14}C]5′-FSBA or its derivatives was eliminated because 5′-FSBA and its derivatives migrate at different rates under the same separation condition. In addition, the radiolabeled fraction (C-28) was absent from the NADH-protected sample. It is, therefore, unlikely that the radioactivity associated with this fraction is due to the unbound ligand or due to nonspecific labeling.

5′-FSBA has been reported to react with amino acids that contain nucleophilic groups, such as cysteine, serine, histidine, lysine, and tryosine (20). Among these potentially reactive amino acids, only the reaction products with tryosine and lysine residues are stable under acidic conditions (21, 22). Because of the instability of the 5′-FSBA labeling in this study, it is

suggested that the affinity probe may react with amino acid residues other than tryosine or lysine in these peptides. The first peptide, ^{146}Ile-Thr-Gly-Gly-Ser-Gly-Ser-Met-Tyr^{155}, contains two serine and two threonine residues, which are potential candidates for reaction with 5′-FSBA. The second peptide, ^{262}Ser-Ile-Pro-Ala-Asp-Asn-Gln-Ile-Lys^{270}, contains one serine which could be the residue modified by 5′-FSBA. The length of the first peptide has been confirmed by fast atom bombardment mass spectral analysis and was probably generated as a result of the contaminating chymotrypsin activity in the trypsin we used, because its last amino acid residue is tyrosine. It has been shown that the nucleotide binding sites of many kinases (23) and dehydrogenases or oxidoreductases (24) contain a so-called "glycine-rich" region, GXGXXG, which maintains the appropriate secondary or tertiary structure for nucleotide binding. This region is usually adjacent to the region where the adenine-ribose-diphosphate moiety of the nucleotide molecule binds. The first peptide (I-T-T-G-G-S-G-S-M-Y) found in the radioactive fraction is a glycine-rich region of the enzyme, although it does not have a GXGXXG type of arrangement. The amino acid sequence of rat liver DT-diaphorase deduced from the cDNA sequence (18, 19) and by direct protein sequencing (14) does not contain the usual GXGXXG sequence. Furthermore, the identified peptide is rich in hydroxy amino acids, which have been suggested to form hydrogen bonds with the phosphate moiety of the nucleotide molecules (25). 5′-FSBA has a structure with the fluorosulfonylbenzoyl group at the 5′-position of the ribose moiety of the molecule. Therefore, it is reasonable to think that 5′-FSBA labels the enzyme near the region where the pyrophosphate group of the nucleotide molecule binds. With these analyses in mind, the peptide I-T-T-G-G-S-G-S-M-Y could be the peptide modified by 5′-FSBA and could be near the region where the pyrophosphate group of the NAD(P)H molecule binds. Interestingly enough, in a recent paper by Prochaska (26), the same region (residues 142–158) was suggested to be a region of pyrophosphate binding for NAD(P)H or FAD, based on a limited sequence homology comparison with rabbit adenylate kinase, rat liver alcohol dehydrogenase, *Drosophilia Dras* 2 oncogene, and human glutathione reductase.

In conclusion, we have presented results demonstrating that 5′-FSBA is an active site-directed reagent for rat liver DT-diaphorase. A stoichiometry of 1 mol of 5′-FSBA bound/mol of active site of the enzyme was determined from inactivation kinetics (Fig. 4) and from incorporation studies using [^{14}C]5′-FSBA (Fig. 5). We found that the irreversible inactivation (Fig. 3) as well as the covalent incorporation could be completely prevented by the presence of NAD(P)H during the incubation. These results indicate that, as expected, 5′-FSBA binds only to the pyridine nucleotide binding site of the enzyme. The potential pyrophosphate binding region of the NAD(P)H binding site is suggested by labeling experiments with [^{14}C]5′-FSBA.

References

1. Ernster, L., M. Ljunggren, and L. Danielson. Purification and some properties of a highly dicoumarol-sensitive liver diaphorase. *Biochem. Biophys. Res. Commun.* **2**:88–92 (1960).
2. Iyanagi, T., and I. Yamazaki. One electron-transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). *Biochim. Biophys. Acta* **216**:282–294 (1970).
3. Iyanagi, T. On the mechanisms of one- and two-electron transfer by flavin enzymes. *Chem. Scr.* **27A**:31–36 (1987).
4. Thor, H., M. T. Smith, G. Hartzell, G. Bellomo, S. A. Jewell, and S. Orrenius. The mechanism of menadione (2-methyl-1,4-naphthoquinone) by isolated hepatocytes: a study of the implications of oxidative stress in intact cells. *J. Biol. Chem.* **257**:12419–12425 (1982).
5. Lind, C., P. Hochstein, and L. Ernster. DT-diaphorase as a quinone reductase: a cellular control device against semiquinone and superoxide radical formation. *Arch. Biochem. Biophys.* **216**:178–185 (1982).
6. Miller, M. G., A. Rodgers, and G. M. Cohen. Mechanisms of toxicity of naphthaquinones to isolated hepatocytes. *Biochem. Pharmacol.* **35**:1177–1184 (1986).
7. Wallin, R., O. Gebhardt, and H. Prydz. NAD(P)H dehydrogenase and its role in vitamin K (2-methyl-3-phytyl-1,4-naphthaquinone-dependent carboxylation reaction. *Biochem. J.* **169**:95–101 (1978).
8. Segura-Aguilar, J. E., C. Lind, O. Nordstrom, and T. Bartfai. Regional and subcellular distribution of DT-diaphorase in the rat brain. *Chem. Scr.* **27A**:55–57 (1987).
9. Kuonen, D. R., N. C. Kemp, and P. J. Roberts. Demonstration and biochemical characterization of rat brain NADPH-dependent diaphorase. *J. Neurochem.* **50**:1017–1025 (1988).
10. Ferrante, R. J., N. W. Kowall, M. F. Beal, E. P. Richardson, Jr., E. D. Bird, and J. M. Martin. Selective sparring of a class of striatal neurons in Huntington's disease. *Science (Wash. D. C.)* **230**:561–563 (1985).
11. Koh, J. Y., S. Peters, and D. W. Chio. Neurons containing NADPH-diaphorase are selectively resistant to quinolinate toxicity. *Science (Wash. D. C.)* **234**:73–76 (1986).
12. Colman, R. F. Affinity labeling of purine nucleotide sites in proteins. *Annu. Rev. Biochem.* **52**:67–92 (1983).
13. Chen, S., M. Haniu, T. Iyanagi, and J. E. Shively. Affinity labeling of the active site of pig liver NADH-cytochrome b_5 reductase by 5′-p-fluorosulfonylbenzoyladenosine. *J. Protein Chem.* **5**:133–145 (1986).
14. Haniu, M., H. Yuan, S. Chen, T. Iyanagi, T. D. Lee, and J. E. Shively. Structure-function relationship of NAD(P)H quinone reductase: characterization of NH_2-terminal blocking group and essential tryosine and lysine residues. *Biochemistry* **27**:6877–6883 (1988).
15. Hawke, D. H., D. C. Harris, and J. E. Shively. Microsequence analysis of peptides and proteins. V. Design and performance of a novel gas-liquid-solid phase instrument. *Anal. Biochem.* **147**:315–330 (1985).
16. Walsh, C. Recent developments in suicide substrates and other active site-directed inactivating agents of specific target enzymes. *Horizons Biochem. Biophys.* **3**:36–81 (1977).
17. Hojeberg, B., K. Blomberg, S. Stenberg, and C. Lind. Biospecific absorption of hepatic DT-diaphorase on immobilized dicoumarol. 1. Purification of cytosolic DT-diaphorase from control and 3-methylcholanthrene-treated rats. *Arch. Biochem. Biophys.* **207**:205–216 (1981).
18. Robertson, J. A., H-C. Chen, and D. W. Nebert. NAD(P)H:menadione oxidoreductase: novel purification of enzyme, cDNA, and complete amino acid sequence, and gene regulation. *J. Biol. Chem.* **261**:15794–15799 (1986).
19. Bayney, R. M., J. A. Rodkey, C. D. Bennett, A. R. H. Lu, and C. B. Pickett. Rat liver NAD(P)H: quinone reductase nucleotide sequence analysis of a quinone reductase cDNA clone and prediction of the amino acid sequence of the corresponding protein. *J. Biol. Chem.* **262**:572–575 (1987).
20. Colman, R. F., P. K. Pal, and J. L. Wyatt. Adenosine derivatives for dehydrogenases and kinases. *Methods Enzymol.* **46**:240–248 (1977).
21. Esch, F. S., and W. S. Allison. Identification of a tyrosine residue at a nucleotide binding site in the β subunit of the mitochondrial ATPase with *p*-fluorosulfonyl-[^{14}C]benzoyl-5′-adenosine. *J. Biol. Chem.* **253**:6100–6106 (1978).
22. Saradambal, K. V., R. A. Bednar, and R. F. Colman. Lysine and tyrosine in the NADH inhibitory site of bovine liver glutamate dehydrogenase. *J. Biol. Chem.* **256**:11866–11872 (1981).
23. Kamps, M. P., S. S. Taylor, and B. M. Sefton. Direct evidence that oncogenic tyrosine kinases and cyclic AMP-dependent protein kinase have homologous ATP-binding sites. *Nature (Lond.)* **310**:589–591 (1984).
24. Hofsteenge, J., J. M. Vereijken, W. J. Weijer, J. J. Beintema, R. F. Wierenga, and J. Drenth. Primary and tertiary structure studies of *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens*. *Eur. J. Biochem.* **113**:141–150 (1980).
25. Proter, T. D., and C. B. Kasper. NADPH-cytochrome P-450 oxidoreductase: flavin mononucleotide and flavin adenine dinucleotide domains evolved from different flavoproteins. *Biochemistry* **25**:1682–1687 (1986).
26. Prochaska, H. J. Purification and crystallization of rat liver NAD(P)H:(Quinone-acceptor) oxidoreductase by Cibacron Blue affinity chromatography: identification of a new and potent inhibitor. *Arch. Biochem. Biophys.* **267**:509–538 (1988).

Send reprint requests to: Shiuan Chen, Division of Immunology, Beckman Research Institute of the City of Hope, Durate, CA 91010.