Thomas, J. L., Myers, R. P., Rosik, L. O., & Strickler, R. C. (1990) *J. Steroid Biochem. 36*, 117–123.
Wierenga, R. K., De Maeyer, M. C. H., & Hol, W. G. J. (1985) *Biochemistry 24*, 1346–1357.
Wierenga, R. K. Terpstra, & Hol, W. G. J. (1986) *J. Mol. Biol. 187*, 101–107.
Witten, J. L., Schaffer, M. H., O'Shea, M., Cook, J. C., Hemling, M. E., & Rinehart, K. L. (1984) *Biochem. Biophys. Res. Commun. 124*, 350–358.
Zhao, H.-F., Simard, J., Labrie, C., Breton, N., Rhêaume, E., Luu-The, V., & Labrie, F. (1989) *FEBS Lett. 259*, 153–157.

Affinity Labeling of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase by 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

Kay J. Rutherfurd, Shiuan Chen, and John E. Shively*
Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010
Received October 18, 1990; Revised Manuscript Received June 4, 1991

ABSTRACT: Incubation of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (5′-FSBA) results in the inactivation of the 3β-hydroxysteroid dehydrogenase enzyme activity 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 prior to an irreversible inactivation reaction. The dissociation constant (K_d) for the initial reversible enzyme–inhibitor complex is estimated at 0.533 mM, with $k_2 = 0.22$ min^{-1}. The irreversible inactivation could be prevented by the presence of NAD$^+$ during the incubation, indicating that 5′-FSBA inactivates the 3β-hydroxysteroid dehydrogenase activity by reacting at the NAD$^+$ binding site. Although the enzyme was inactivated by incubation with 5′-FSBA, no incorporation of the inhibitor was found in labeling studies using 5′-[*p*-(fluorosulfonyl)benzoyl][^{14}C]adenosine. However, the inactivation of 3β-hydroxysteroid dehydrogenase activity caused by incubation with 5′-FSBA could be completely reversed by the addition of dithiothreitol. This indicates the presence of at least two cysteine residues at or in the vicinity of the NAD$^+$ binding site, which may form a disulfide bond catalyzed by the presence of 5′-FSBA. The intramolecular cysteine disulfide bridge was found between the cysteine residues in the peptides ^{274}EWGFCLDSR^{282} and ^{18}IICLLVEEK^{26}, by comparing the [^{14}C]iodoacetic acid labeling before and after recovering the enzyme activity upon the addition of dithiothreitol.

The 3β-hydroxysteroid dehydrogenase/steroid isomerase enzyme complex (EC 1.1.1.51, EC 5.3.3.1) catalyzes consecutive steps in the conversion of pregnenolone to progesterone. NAD$^+$ is the cofactor for the dehydrogenase activity, and this nucleotide has also been shown to be a potent allosteric activator of the steroid isomerase activity (Ishii-Ohba et al., 1986a,b, 1987; Brandt & Levy, 1989; Thomas et al., 1988). Therefore, NAD$^+$ plays an important role in both the dehydrogenation and isomerization reactions catalyzed by this enzyme complex.

To date, the nucleotide binding site of 3β-hydroxysteroid dehydrogenase/steroid isomerase has not been identified, although the primary structure of the enzyme has been determined by cloning (Luu-The et al., 1989; Zhao et al., 1989) and by protein sequencing (Rutherfurd et al., 1991). Affinity labeling studies can provide information as to the importance and role of regions in the active site of the enzyme. Previous studies have shown that 5′-[(fluorosulfonyl)benzoyl]adenosine (5′-FSBA), an adenine analogue that has proven to be a useful affinity label for studies of many adenine nucleotide and nicotinamide-dependent enzymes (Colman, 1983; Chen et al., 1986; Liu et al., 1989), inactivates rat testicular and adrenal 3β-hydroxysteroid dehydrogenase activities (Ishii-Ohba et al., 1986a,b). The modified residue(s) in these enzymes were not determined (Ishii-Ohba et al., 1986a,b). As a first step in determining the structure of the NAD$^+$ binding site of 3β-hydroxysteroid dehydrogenase/steroid isomerase, an affinity labeling study using 5′-FSBA has been carried out in this laboratory. Results presented here show that 5′-FSBA can be used to modify the NAD$^+$ binding site of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, resulting in the inactivation of the 3β-hydroxysteroid dehydrogenase activity. Kinetically, 5′-FSBA fulfills all of the criteria of an affinity label for 3β-hydroxysteroid dehydrogenase/steroid isomerase, but our data suggest that the inactivation caused by 5′-FSBA is due to the formation of a disulfide bond that is catalyzed by 5′-FSBA.

Experimental Procedures

Materials. 5′-FSBA, NAD$^+$, and trypsin (TPCK-treated) were obtained from Sigma Chemical Co. (St. Louis, MO). 5′-[(Fluorosulfonyl)benzoyl][*adenine*-8-^{14}C]adenosine (53.6 mCi/mmol) was obtained from New England Nuclear Corp. (Boston, MA). [1-^{14}C]Iodoacetic acid (6.25 mCi/mmol) was obtained from ICN Biomedicals, Inc. (Costa Mesa, CA).

Enzyme Preparation. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was purified to homogeneity from bovine adrenal glands by a combination of ion-exchange chromatography with DEAE Toyopearl 650S and adsorption

† This work was supported in part by research grants from the National Institutes of Health (GM 37297 and HD 14900).

0006-2960/91/0430-8116$02.50/0

chromatography with hydroxylapatite, as described previously (Rutherfurd et al., 1991).

Enzyme Assay. 3β-Hydroxysteroid dehydrogenase enzyme activity was determined spectrophotometrically as reported previously (Rutherfurd et al., 1991).

Affinity Labeling of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase with 5′-FSBA. In order to achieve an effective labeling of the enzyme with 5′-FSBA, the NAD^+, which was added during the purification procedure, was removed from the enzyme preparation prior to use by Sephadex G-25 syringe-column centrifugation (Chen & Guillory, 1981). The reaction of 3β-hydroxysteroid dehydrogenase/steroid isomerase with either 5′-FSBA or the control compound 4-(fluorosulfonyl)benzoic acid (BSF) was carried out at 25 °C in 100 mM potassium phosphate buffer, 20% glycerol, and 0.1 mM EDTA containing 10% dimethyl sulfoxide (DMSO)[1] at pH 7.5. Ten percent dimethyl sulfoxide was included as it was required to maintain the solubility of 5′-FSBA over the incubation period and had no effect on the 3β-hydroxysteroid dehydrogenase activity. The extent of inactivation was monitored by measuring the residual enzyme activity at given time intervals.

Determination of the Stoichiometry of 5′-FSBA Labeling. 3β-Hydroxysteroid dehydrogenase/steroid isomerase (112 μg/mL) was incubated with [^{14}C]-5′-FSBA (0–0.2 mM) under the conditions described above. After 20 min of incubation, the reaction mixture was cooled to 4 °C, and a 40-μL aliquot was removed from each mixture for the determination of residual enzyme activity. The remainder of the samples were each denatured in the presence of 1% SDS by incubation in a boiling water bath for 3 min. The samples were then electrophoresed on a 7.5% SDS–polyacrylamide gel (Laemmli, 1970). The protein bands, visualized by staining with Coomassie blue, were excised from the gel, and the gel pieces were dissolved in 0.1 mL of 30% hydrogen peroxide. The bound radioactivity was then evaluated.

The amount of radioactivity associated with the enzyme was also determined by isolating the modified enzyme by reverse-phase HPLC with a Brownlee C-4 cartridge column (4.6 × 30 mm). The protein was well separated from the free analogues, and recoveries were greater than 90%.

The 5′-FSBA-treated protein was also separated from the free analogues by gel filtration on a Sephadex G-25–50 column (7 × 100 mm) in 100 mM potassium phosphate, pH 7.5, and 20% glycerol. Fractions were manually collected and the bound radioactivity was evaluated on an aliquot from each fraction.

Specific Labeling of the Cysteine Residues at the NAD^+ Binding Site of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase. One sample of 3β-hydroxysteroid dehydrogenase/steroid isomerase containing NAD^+ (molar ratio of enzyme to NAD^+, 1:1.5) and another containing 1 mM 5′-FSBA were incubated in 100 mM potassium buffer (pH 7.5), 20% glycerol, and 0.1 mM EDTA, for 1 h at 25 °C. Following incubation, unlabeled iodoacetic acid (3-fold molar excess over thiol groups) was added to both samples and incubated under nitrogen in the dark at 4 °C for 24 h. This step ensures all the available unprotected cysteine residues are carboxymethylated with cold iodoacetic acid. The resulting reaction mixtures were then subjected to Sephadex G-25 syringe-column centrifugation to remove the NAD^+, 5′-FSBA, and iodoacetic acid. To a portion of the 5′-FSBA-treated 3β-hydroxysteroid dehydrogenase/steroid isomerase was added DTT (molar ratio of enzyme to DTT, 1:3.5) to reduce any disulfide bonds that may have formed. The three reaction mixtures were then each alkylated with [1-^{14}C]-iodoacetate under nitrogen in the dark at 4 °C for 24 h. Alkylation was terminated by the addition of 2-mercaptoethanol (100 mmol). The samples were then subjected to reverse-phase HPLC, which was performed on a Brownlee C-4 column (4.6 × 30 mm), to remove excess [^{14}C]iodoacetic acid and 2-mercaptoethanol. Following lyophilization, the radiolabeled enzyme was again subjected to alkylation under denaturing conditions, to ensure that any cysteine residues which were unavailable for alkylation in the native enzyme would be reacted with cold iodoacetic acid. The enzyme samples were each dissolved in 1 mL of 6 M guanidine hydrochloride and 0.25 M Tris-HCl (pH 8.5) and sparged with nitrogen prior to the addition of 0.15 mg of DTT. Reduction was allowed to occur for 2 h prior to the addition of a 3-fold molar excess of unlabeled iodoacetic acid over the total thiol content. Alkylation was performed under nitrogen in the dark at 25 °C for 45 min before termination of the reaction by the addition of 2-mercaptoethanol (100 mmol). The carboxymethylated protein was then desalted by reverse-phase HPLC as described above and lyophilized prior to tryptic digestion.

Tryptic Digestion. The lyophilized protein, which had been treated with unlabeled and labeled iodoacetic acid as described above, was dissolved in 20 μL of HFA, followed by 1 mL of 100 mM ammonium bicarbonate. The pH was adjusted to 8.5 prior to the addition of TPCK-treated trypsin in an enzyme:substrate ratio of 1:100 (w/w). Digestion was allowed to proceed for 18 h at 37 °C.

Analysis and Separation of Fragments by HPLC. Tryptic peptides were separated by reverse-phase HPLC performed on a Vydac C-18 column (4.6 × 250 mm), with a 2-h linear gradient from 100% solvent I (0.1% TFA) to 100% solvent II (0.1% TFA and 90% acetonitrile) at a flow rate of 0.5 mL/min. Peptides were detected by absorbance at 214 nm and were manually collected. Radiolabeled peptides were detected by measurement of ^{14}C radioactivity from 20 μL of each fraction.

Microsequence Analysis. Approximately 50–200 pmol of peptide was subjected to automated Edman degradation, performed on a gas-phase peptide/protein microsequencer (Hawke et al., 1985). The phenylthiohydantoin amino acid derivatives were identified by reverse-phase HPLC on a Beckman Ultrasphere ODS column (2.0 × 250 mm) and were quantitated by integration with Perkin-Elmer LIMS software.

Mass Spectral Analysis. Positive-ion fast-atom bombardment (FAB) mass spectra were obtained by using a JEOL HX-100HF high-resolution, double-focusing, magnetic-sector mass spectrometer operating at 5 kV accelerating potential and a nominal resolution of 3000. Sample ionization was accomplished by using a 6-keV Xe atom beam. A JEOL DA5000 data system was used to control instrument parameters and collect the spectral data. Peptides were dissolved in 2 μL of DMSO and added to 1 μL of sample matrix [dithiothreitol–dithioerythritol (5:1) (Witten et al., 1984) and camphorsulfonic acid (6 mM) (DePauw et al., 1984)] on a 1.5 × 6 mm stainless steel sample stage.

Results

Inactivation of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase Activity by 5′-FSBA. If 5′-FSBA is indeed binding at the active site of the enzyme, then there are specific

[1] Abbreviations: HPLC, high-performance liquid chromatography; HFA, hexafluoroacetone trihydrate; TFA, trifluoroacetic acid; ODS, octadecylsilane; FAB, fast atom bombardment; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); SDS, sodium dodecyl sulfate.

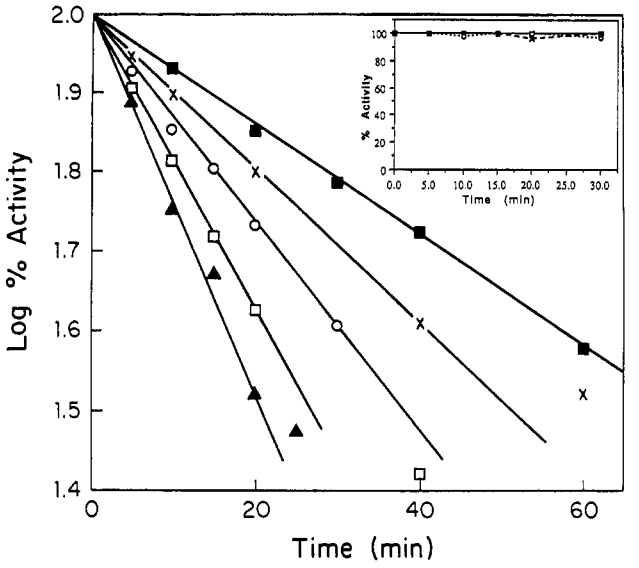


FIGURE 1: Inactivation of 3β-hydroxysteroid dehydrogenase enzyme by 5′-FSBA. Bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase (163 μg/mL) was incubated with varying concentrations of 5′-FSBA (▲, 0.15 mM; □, 0.113 mM; ○, 0.075 mM; ×, 0.0563 mM; ■, 0.0375 mM) at 25 °C in 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, and 10% dimethyl sulfoxide. At the times indicated, aliquots were withdrawn and assayed for 3β-hydroxysteroid dehydrogenase activity. (Inset) Bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase (163 μg/mL) was incubated with 0.075 mM BSF (○, ---), without BSF (■, —), and with 0.075 mM BSF and 7.5 mM NAD^+ (×, ---) at 25 °C in 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol, 0.1 mM EDTA, and 10% dimethyl sulfoxide. At the times indicated, aliquots were withdrawn and assayed for 3β-hydroxysteroid dehydrogenase activity.

kinetic criteria that must hold true. First, the time-dependent inactivation of bovine adrenal 3β-hydroxysteroid dehydrogenase activity upon incubation with 5′-FSBA [in 100 mM potassium phosphate buffer (pH 7.5), containing 20% glycerol, 0.1 mM EDTA, and 10% dimethyl sulfoxide at 25 °C] should exhibit pseudo-first-order kinetics, as is indicated by the semilogarithmic plot of residual enzyme activity versus time of incubation (Figure 1). The observed initial rate constants for inactivation (k_{obs}) at 0.15, 0.113, 0.075, 0.0563, and 0.0375 mM 5′-FSBA are 0.0533, 0.0433, 0.0308, 0.0239, and 0.0163 min^{-1}, respectively. Second, a double-reciprocal plot of $1/k_{obs}$ versus 1/[5′-FSBA] should yield a straight line with a positve y-intercept (Figure 2), indicative of the reversible binding of the inhibitor prior to the irreversible inactivation:

$$E + 5'\text{-FSBA} \overset{K_d}{\rightleftharpoons} E\cdots 5'\text{-FSBA} \xrightarrow{k_2} E^*$$

where E* = inactive complex. From the y-intercept of this double-reciprocal plot, the value of the first-order rate constant for inactivation, $k_2 = 0.22$ min^{-1}, can be calculated, and from the x-intercept the dissociation constant for 5′-FSBA from the reversible complex $K_d = 0.533$ mM can be calculated. Enzyme incubated in the presence of 10% dimethyl sulfoxide but not 5′-FSBA maintained full activity under the same conditions. Enzyme incubated with 0.075 mM BSF instead of 5′-FSBA also maintained the same activity as the control without BSF under the same conditions (Figure 1).

Effect of NAD^+ on the Inactivation of Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase Enzyme Activity by 5′-FSBA. If 5′-FSBA modifies or occupies the NAD^+ binding site of the enzyme, then the true substrate, NAD^+, should provide specific protection against inactivation by 5′-FSBA. NAD^+ was found to protect against the inactivation of 3β-hydroxysteroid dehydrogenase activity induced by incubation

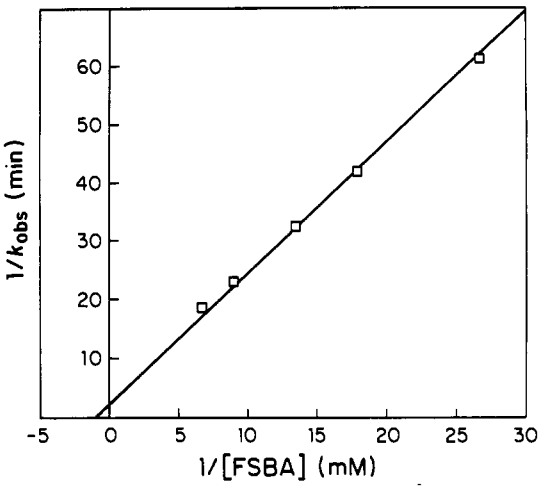


FIGURE 2: Dependence of pseudo-first-order rate constant of inactivation of 3β-hydroxysteroid dehydrogenase activity on 5′-FSBA concentration. The pseudo-first-order rate constants for the loss of 3β-hydroxysteroid dehydrogenase enzyme activity, k_{obs}, were calculated from curves as shown in Figure 1.

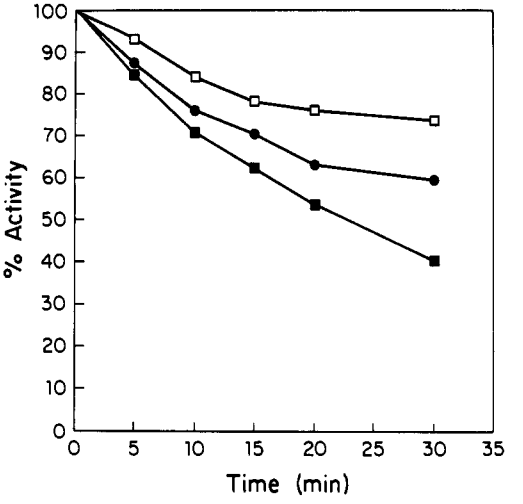


FIGURE 3: Protection from 5′-FSBA inactivation of 3β-hydroxysteroid dehydrogenase activity by NAD^+. 3β-Hydroxysteroid dehydrogenase/steroid isomerase (163 μg/mL) was incubated with 0.075 mM 5′-FSBA and protecting NAD^+ at the concentrations indicated (■, 0 mM NAD^+; ●, 5 mM NAD^+; □, 10 mM NAD^+). The 3β-hydroxysteroid dehydrogenase activity of the control, in the absence of 5′-FSBA, was taken as 100%.

with 5′-FSBA in a concentration-dependent manner, as is shown in Figure 3. Enzyme incubated with BSF and NAD^+ maintained the same activity as the control without BSF (Figure 1). These results indicate that the 5′-FSBA modification does indeed occur at the nicotinamide nucleotide binding site of 3β-hydroxysteroid dehydrogenase/steroid isomerase.

Stoichiometry of the Reaction of 5′-FSBA with Bovine Adrenal 3β-Hydroxysteroid Dehydrogenase/Steroid Isomerase. The stoichiometry of 3β-hydroxysteroid dehydrogenase/steroid isomerase inactivation by 5′-FSBA was determined by inactivation kinetics. Since the inactivation of the enzyme by 5′-FSBA has been shown to proceed according to pseudo-first-order kinetics with respect to the 5′-FSBA concentration, the enzyme inactivation by this compound can be described by

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

where n is the number of 5′-FSBA molecules reacting per active site. When the 5′-FSBA concentration is much greater

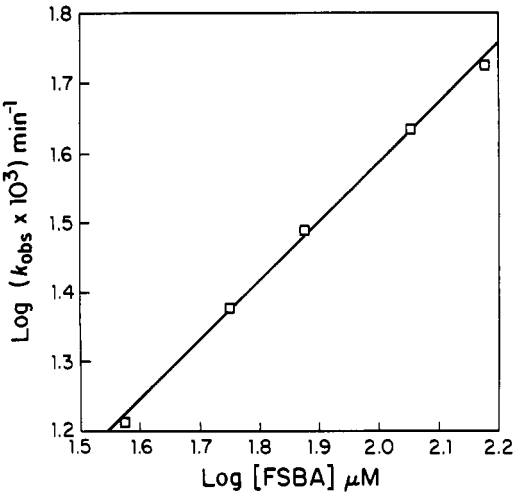


FIGURE 4: Kinetic determination of the stoichiometry of inactivation of 3β-hydroxysteroid dehydrogenase activity by 5′-FSBA. The pseudo-first-order rate constant of inactivation of 3β-hydroxysteroid dehydrogenase activity, k_{obs}, was obtained from Figure 1.

than that of the enzyme, such as under the experimental conditions used here, then the rate of enzyme inactivation is

$$d[E]/dt = k[E][5'\text{-FSBA}]^n$$

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

$$k_{obs} = k[5'\text{-FSBA}]^n$$

and

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

The latter equation may be used to determine the value of the inactivation stoichiometry, n. The slope of the line obtained from a double-logarithmic plot of k_{obs} versus [5′-FSBA] (Figure 4) is 0.9, indicating that 3β-hydroxysteroid dehydrogenase/steroid isomerase was inactivated by the reaction of 1 mol of 5′-FSBA/mol of the NAD^+ binding site.

The specificity of the affinity label can also be shown by the stoichiometry of the labeling. The covalent incorporation of [^{14}C]-5′-FSBA into 3β-hydroxysteroid dehydrogenase/steroid isomerase was measured by three different methods. First, the incorporation was investigated by separating the modified enzyme by SDS–polyacrylamide gel electrophoresis, as described under Experimental Procedures. Using this method, we were unable to detect any significant incorporation of [^{14}C]-5′-FSBA into 3β-hydroxysteroid dehydrogenase/steroid isomerase (less than 0.01 mol of [^{14}C]-5′-FSBA/mol of enzyme). The extent of incorporation was also determined by isolating the modified enzyme by reverse-phase HPLC; again, less than 0.01 mol of [^{14}C]-5′-FSBA was incorporated per mol of enzyme. Even under the less harsh conditions of gel filtration under nondenaturing conditions, significant incorporation of [^{14}C]-5′-FSBA into the enzyme still could not be detected.

Interaction of 5′-FSBA with Enzyme Thiols. It has been reported that 5′-FSBA reacts with amino acids containing nucleophilic groups, such as cysteine, serine, histidine, lysine, and tyrosine (Colman et al., 1977). Of these potentially reactive amino acids, only the reaction products with tyrosine and lysine have been found to be stable under acidic conditions (Esch & Allison, 1978; Saradambal et al., 1981). Therefore, if a residue other than a tyrosine or lysine were involved, this would explain the lack of [^{14}C]-5′-FSBA incorporation into the enzyme obtained following separation by reverse-phase HPLC. This does not, however, explain the lack of incorporation found by SDS–polyacrylamide gel electrophoresis or by gel filtration. A similar situation has been documented for pyruvate kinase (Annamalai & Colman, 1981), where 5′-FSBA causes inactivation of the enzyme, but the enzyme could not be isolated with the [^{14}C]-5′-FSBA covalently bound. This inactivation was found to be due to the formation of an intramolecular cystine disulfide, which caused the subsequent displacement of the label.

Table I: Reactivation of 3β-Hydroxysteroid Dehydrogenase Activity with Dithiothreitol, following Inactivation by Incubation with 5′-FSBA[a]

treatment		enzyme activity (nmol/min)
−FSBA	−DTT	13.69
+FSBA	−DTT	5.00
−FSBA	+DTT	14.20
+FSBA	+DTT	13.70

[a] 3β-Hydroxysteroid dehydrogenase/steroid isomerase (150 μg) was incubated in the presence or absence of 0.15 mM 5′-FSBA for 20 min as described under Experimental Procedures. Aliquots were then assayed for 3β-hydroxysteroid dehydrogenase activity in the presence and absence of 10 mM DTT. Values are the average of duplicate assays.

On the basis of the assumption that such a modification may also be occurring in the case of bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, the inactivation should be reversed by treating the 5′-FSBA-modified enzyme with an excess of DTT. The addition of DTT to the inactivated enzyme resulted in a rapid and almost complete regain of 3β-hydroxysteroid dehydrogenase activity (Table I), thus implying the existence of two or more cysteine residues at or near the NAD^+ binding site of the bovine adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, which form a disulfide bond resulting in the inactivation of the 3β-hydroxysteroid dehydrogenase activity. Attempts to quantitate the loss of total free thiols of enzyme before and after treatment with 5′-FSBA, by titration with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), indicated that there was a decrease in the number of titratable cysteine residues following treatment with 5′-FSBA. However, due to problems in obtaining the expected theoretical number of cysteine residues in the unreacted enzyme, even in the presence of guanidine hydrochloride or SDS, the absolute number of cysteine residues being titrated before and 5′-FSBA treatment was uncertain. The disulfide bond we have proposed cannot be present in the native protein; otherwise the enzyme would be inactive under the assay conditions used.

Identification of the Cysteine Residues at the NAD^+ Binding Site. 3β-Hydroxysteroid dehydrogenase/steroid isomerase was first S-alkylated with unlabeled iodoacetic acid under native conditions in the presence of a 1.5 molar excess of NAD^+. The NAD^+ and iodoacetic acid were then removed by gel filtration, and the enzyme was S-alkylated with [^{14}C]iodoacetic acid under native conditions, and later with unlabeled iodoacetic acid under denaturing conditions. Following tryptic digestion, the peptides containing the specifically radiolabeled sulfhydryl groups were isolated by reverse-phase HPLC (Figure 5A). Three radiolabeled fractions (N-85, N-91, and N-95) were detected from the NAD^+-protected enzyme. The sequences of the peptides found in fractions N-85, N-91, and N-95 are shown in Table II. The amount of radioactivity present in fractions N-85, N-91, and N-95 was approximately 28%, 27%, and 17%, respectively, of the amount in original modified protein (27 324 cpm).

Fraction N-85 contained a single peptide, the partial sequence of which is shown in Table II. This corresponds to residues 70–78 of the bovine adrenal sequence. A (carboxymethyl)cysteine derivative was detected at cycle 3 of the sequence; however, the amount of radioactivity was insufficient to verify the position of the label at this residue. Fraction N-91 contained a mixture of two peptides, which could not be

Table II: Sequence Analysis of Cysteine-Containing Peptides Protected by NAD^+

	peptide N-85		peptide N-91		peptide N-95	
residue no.	residue	pmol	residue	pmol	residue	pmol
1	Gly	35	Glu	17	Leu	112
2	Ala	21	Trp	12	Thr	23
3	Cys	5	Gly	12	Leu	109
4	Gln	5	Phe	16	Leu	102
5	Gly	8	Cys	7	Glu	62
6	Thr	10	Leu	16	Gly	47
7	Ser	11	Asp	7	Asp	46
8	Val	7	Ser	6	Ile	47
9	Val	14	Arg	4	Leu	72
10					Asp	42
11					Glu	33
12					Gln	30
13					Cys	14
14					Leu	16
15					Lys	4

separated even by rechromatography. Sequence analysis of the peptides showed that both contained a (carboxymethyl)-cysteine derivative; however, the radioactivity was associated with only one of the cysteines. The sequence of the modified peptide (Table II), corresponds to residues 274–282 of the bovine adrenal enzyme (Rutherfurd et al., 1991). The (carboxymethyl)cysteine derivative was detected at cycle 5 of the sequence, along with the radioactivity. Fraction N-95 contained a single peptide with the sequence shown in Table II, corresponding to residues 55–69 of the bovine sequence. At cycle 13 a (carboxymethyl)cysteine derivative was detected, but the position of the radioactivity could not be confirmed for this sample due to a fraction collector malfunction.

Identification of 5′-FSBA-Protected Cysteine Residues. Following inhibition of 3β-hydroxysteroid dehydrogenase by 94% upon incubation with 5′-FSBA, the enzyme preparation was alkylated under native conditions with cold iodoacetic acid as described under Experimental Procedures. The protecting reagent was then removed by gel filtration, and the enzyme was alkylated with [^{14}C]iodoacetic acid under native conditions and finally with cold iodoacetic acid under denaturing conditions following the removal of the excess hot reagent. Following tryptic digestion and reverse-phase HPLC, three radiolabeled fractions (F-69, F-90, and F-97), were detected from the 5′-FSBA-protected enzyme (Figure 5B). The radioactivity present in the fractions were approximately 39%, 36%, and 11% of the original modified protein (18 967 cpm), for F-69, F-90, and F-97, respectively.

Fraction 69 contained a single peptide with sequence shown in Table III. This peptide corresponds to residues 310–316 of the bovine enzyme and is different from the peptides labeled

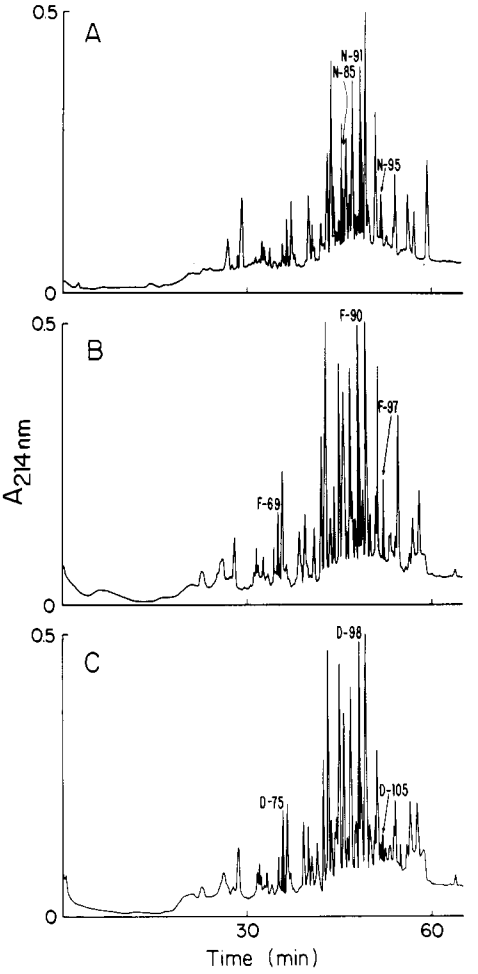


FIGURE 5: Peptide mapping of tryptic peptides derived from (A) NAD^+-protected 3β-hydroxysteroid dehydrogenase/steroid isomerase, (B) 5′-FSBA-treated 3β-hydroxysteroid dehydrogenase/steroid isomerase, and (C) 5′-FSBA- and DTT-treated 3β-hydroxysteroid dehydrogenase/steroid isomerase, each alkylated with unlabeled and ^{14}C-labeled iodoacetic acid. The tryptic peptides from each digest were applied to a Vydac C-18 column (4.6 × 250 mm) and eluted with a linear gradient from 100% solvent I (0.1% TFA) to 100% solvent II (0.1% TFA and 90% acetonitrile) at a flow rate of 0.5 mL/min over 120 min.

in the NAD^+-protected enzyme. A (carboxymethyl)cysteine derivative along with the radioactivity was detected at cycle 4 of the peptide. Fraction F-90 contained a mixture of two peptides, which upon rechromatography were still not completely separated; both were found to be present in approximately equal amounts, and both contained a (carboxy-

Table III: Sequence Analysis of Cysteine-Containing Peptides Protected by 5′-FSBA

	peptide F-69		peptide F-90a		peptide F-90b		peptide F-97	
residue no.	residue	pmol	residue	pmol	residue	pmol	residue	pmol
1	Tyr	11	Glu	72	Ile	65	Leu	158
2	Asn	15	Trp	35	Ile	58	Thr	23
3	Pro	13	Gly	44	Cys	27	Leu	171
4	Cys	6	Phe	63	Leu	56	Leu	226
5	Phe	23	Cys	23	Leu	65	Glu	114
6	Asn	12	Leu	82	Val	50	Gly	88
7	Arg	2	Asp	25	Glu	32	Asp	88
8			Ser	4	Glu	51	Ile	126
9			Arg	4	Lys	8	Leu	114
10							Asp	68
11							Glu	60
12							Gln	35
13							Cys	14
14							Leu	26
15							Lys	12

Table IV: Sequence Analysis of Cysteine-Containing Peptides Protected by 5′-FSBA, with Subsequent DTT Treatment

	peptide D-75		peptide D-98a		peptide D-98b		peptide D-105	
residue no.	residue	pmol	residue	pmol	residue	pmol	residue	pmol
1	Tyr	69	Glu	56	Ile	27	Leu	72
2	Asn	76	Trp	27	Ile	25	Thr	11
3	Pro	60	Gly	68	Cys	11	Leu	67
4	Cys	25	Phe	54	Leu	22	Leu	92
5	Phe	73	Cys	27	Leu	35	Glu	40
6	Asn	57	Leu	72	Val	22	Gly	20
7	Arg	13	Asp	26	Glu	13	Asp	30
8			Ser	3	Glu	24	Ile	30
9			Arg	3	Lys	6	Leu	25
10							Asp	31
11							Glu	16
12							Gln	9
13							Cys	4
14							Leu	9
15							Lys	4

methyl)cysteine derivative. Radioactivity was found to be associated with both of the cysteine residues, indicating the incorporation of the label into both peptides. The sequences of both the labeled peptides (F-90a and F-90b) are shown in Table III. Peptide F-90a corresponds to residues 274–282 of the bovine enzyme and is identical with peptide N-91, which was labeled in the NAD^+-protected enzyme. Peptide F-90b corresponds to residues 18–26 of the bovine enzyme and is identical with the unlabeled peptide that was copurified and separated in fraction N-91 from the NAD^+-protected enzyme. Peptide F-90a and F-90b contained (carboxymethyl)cysteine derivatives and associated radioactivity at cycles 5 and 3 of the sequence respectively. Fraction F-97 contained a single peptide, with sequence (Table III) identical with that of the NAD^+-protected peptide N-95. A (carboxymethyl)cysteine derivative was detected at cycle 13, along with the associated radioactivity.

Identification of 5′-FSBA-Protected Cysteine Residues following DTT Treatment. The enzyme was alkylated as described previously, except that following the first alkylation in the presence of 1 mM 5′-FSBA, DTT was added to reduce any disulfide bonds that may have formed.

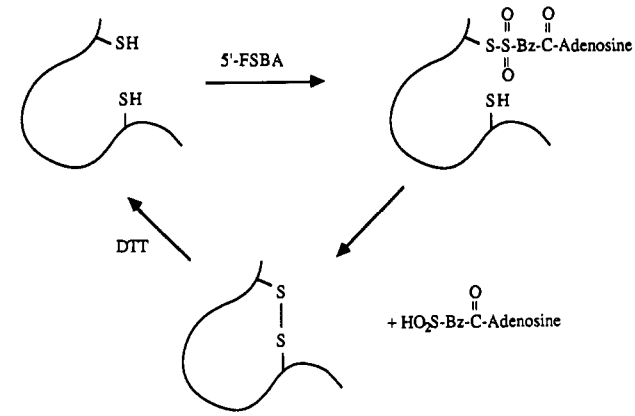
Following reverse-phase HPLC of the tryptic digest (Figure 5C), three radiolabeled fractions (D-75, D-98, and D-105) were detected from the FSBA-protected and DTT-treated enzyme. The radioactivity present in these fractions was 21%, 45%, and 11% of the amount in original modified enzyme (53 927 cpm) for D-75, D-98, and D-105, respectively.

Fraction D-75 contained a single peptide with the sequence shown in Table IV. This is identical with peptide F-69 from the 5′-FSBA-treated enzyme. The (carboxymethyl)cysteine derivative and the radioactivity were detected at cycle 4 of the peptide. Fraction D-98 contained a mixture of two peptides that could not be completely separated upon rechromatography. Both peptides contained (carboxymethyl)cysteine derivatives, and both exhibited the associated radioactivity. The sequences of the two radiolabeled peptides (D-75a and D-75b) are shown in Table IV. Peptide D-75a, with a cysteine at cycle 5 of the sequence, is identical with radiolabeled peptides N-91 and F-90a. Peptide D-75b has a cysteine residue at cycle 3 and is identical with peptide F-90b. Fraction D-105 also contained a single peptide with sequence (Table IV) identical with N-95 and F-95. Again the (carboxymethyl)cysteine derivative and the associated radioactivity were detected at cycle 13.

Discussion

Our results indicate that 5′-FSBA is an NAD^+ analogue for 3β-hydroxysteroid dehydrogenase/steroid isomerase.

Scheme I



Control reactions with 4-(fluorosulfonyl)benzoic acid did not lead to inhibition of the enzyme; therefore, 5′-FSBA is not randomly labeling reactive nucleophiles on the surface of the protein, but rather its action is directed toward the NAD^+ binding site by the presence of the adenine moiety. 5′-FSBA fulfills the criteria of an affinity label in that it is capable of providing substantial, concentration-dependent, saturatable enzyme inactivation, which can be prevented by the inclusion of the true substrate, NAD^+. On the basis of these results, it is highly likely that the 5′-FSBA-dependent loss of 3β-hydroxysteroid dehydrogenase activity reflects a modification that occurs either at the NAD^+ binding site or in the near vicinity. The lack of incorporation of [^{14}C]-5′-FSBA into the protein, along with the fact that DTT fully reverses the inactivation, implies that the formation of a disulfide bond catalyzed by 5′-FSBA is responsible for the inativation of 3β-hydroxysteroid dehydrogenase activity upon incubation with 5′-FSBA. The mechanism for the formation of a disulfide bond is shown in Scheme I. Here, one cysteine reacts with 5′-FSBA to form an intermediate (sulfonylbenzoyl)-adenosylated cysteine. This is followed by an attack from a neighboring cysteine and subsequent displacement of the (sulfonylbenzoyl)adenosine to form an intramolecular disulfide. This mechanism is not without precedence: Parsons et al. (1965) utilized the reaction of thiosulfates with thiols as a synthetic route for the production of disulfides. Similar mechanisms have been proposed for the inactivation of rabbit muscle pyruvate kinase (Tomich et al., 1981; Annamalai & Colman, 1981), myosin subfragment 1 (Togashi & Reisler, 1982), and rabbit muscle phosphofructokinase (Ogilvie, 1983), by (*p*-fluorosulfonyl)benzoyl nucleoside derivatives. The identity of the cysteine residues involved in the disulfide formation have been established in some of these cases (Gomi

Table V: Extent of Radiolabel Incorporation into the Labeled Peptides Isolated from the NAD+-Protected, 5′-FSBA-Treated, and 5′-FSBA- and DTT-Treated Enzyme

peptide sequence	peptide name	cpm/100 pmol	ratio
^{70}GACQGTSVV^{78}	N-85	IN[a]	IN
^{55}LTLLEGDILDEQCLK^{69}	N-95	ND	ND
	F-97	29	1
	D-105	30	1
^{310}YNPCFNR^{316}	F-69	433	1
	D-75	491	1.1
^{274}EWGFCLDSR^{282}	N-91	88	2.9
	F-90a	31	1
	D-98a	465	15.0
^{18}IICLLVEEK^{26}	F-90b	28	1
	D-98b	430	15.4

[a] IN = insufficient counts to verify position; ND = not determined due to fraction collector malfunction.

et al., 1986). In the present investigation we attempted to isolate the cysteine-containing peptides involved in this disulfide.

Selective labeling of the cysteine residues at the NAD+ binding site was carried out by first protecting these residues by incubation with either NAD+ or 5′-FSBA and then carboxymethylating with cold iodoacetic acid. The protecting reagents were then removed by gel filtration, and DTT was added to 1 mg of the 5′-FSBA-treated enzyme to reduce any disulfides that may have formed. The three differently treated mixtures were then carboxymethylated with [^{14}C]iodoacetic acid. By this method three labeled peptides were isolated and sequenced for the NAD+-treated enzyme and four each for the 5′-FSBA- and the 5′-FSBA- and DTT-treated enzymes. Two peptides (^{55}LTLLEGDILDEQCLK^{69} and ^{274}EWGFCLDSR^{282}) were labeled in all three preparations, indicating that the 5′-FSBA is indeed reacting at the NAD+ binding site and localizing these two peptides to the NAD+ binding site. Comparison of the 5′-FSBA- and the 5′-FSBA- and DTT-treated enzyme showed that two peptides, ^{274}EWGFCLDSR^{282} and ^{18}IICLLVEEK^{26}, exhibited increased ^{14}C incorporation following the DTT treatment. These results indicate that the inactivation of 3β-hydroxysteroid dehydrogenase activity by 5′-FSBA is caused by the formation of an intramolecular cystine disulfide bridge and indicates the residues involved in the disulfide bond.

Table V shows the labeled peptides and the extent of radiolabel incorporated into each peptide as determined from the counts detected in the (carboxymethyl)cysteine fraction following sequencing. The yields were calculated from the first residue of each peptide in order to allow comparison of the radioactivity associated with each identical peptide from the different treatments. Further comparisons between the peptides cannot be made since losses that occur during the sequencing process result in the number of counts being lower than expected the more cycles occurring before the (carboxymethyl)cysteine residue.

The peptide ^{70}GACQGTSVV^{78} was found to be labeled only in the NAD+-protected enzyme, indicating that it is not involved in disulfide bond formation and reflecting the difference in the tertiary structure of NAD+ and its analogue 5′-FSBA. This peptide corresponds to residues 70–78 of the bovine sequence with the cysteine at residue 72. In the human placental enzyme this residue is also a cysteine (Luu-The et al., 1989). Peptide ^{55}LTLLEGDILDEQCLK^{69} was found to be labeled in all three preparations and in approximately equal amounts. This localizes this peptide to the NAD+ binding site and also indicates that it is not involved in disulfide bond formation. This peptide corresponds to residues 55–69 of the bovine protein, with the cysteine at residue 67. In human placental 3β-hydroxysteroid dehydrogenase/steroid isomerase this cysteine has been substituted for a phenylalanine residue. Peptide ^{310}YNPCFNR^{316} was found to be labeled only in the 5′-FSBA- and the 5′-FSBA- and DTT-treated enzymes. The extent of incorporation into the peptide was approximately equal in each case, indicating that this peptide is not involved in the disulfide bond. It is likely that the selective protection toward residue 313 by 5′-FSBA and by 5′-FSBA and DTT is due to the difference in size and conformation of the 5′-FSBA compared to NAD+. We have shown in this study that 5′-FSBA does occupy the NAD+ binding site; therefore, it is likely that cysteine 313, while not residing within the NAD+ binding site, is located not far from it. This peptide corresponds to residues 310–316 of the bovine protein, with the cysteine at position 313. Human placental 3β-hydroxysteroid dehydrogenase has a proline residue substituted for the cysteine in this position.

The peptide ^{274}EWGFCLDSR^{282} was found to be labeled in all three enzyme preparations, indicating its location at the NAD+ binding site. The ratio of the counts in this peptide showed a dramatic 15-fold increase in incorporation of radiolabel into the 5′-FSBA- and DTT-treated enzyme over the 5′-FSBA-treated enzyme. This implies that this cysteine is involved in the disulfide bond formation. The location of this peptide is at residues 274–282 of the bovine sequence, with the cysteine at position 278. At this position in the human placental sequence an arginine residue has been substituted. The peptide ^{18}IICLLVEEK^{26} was labeled in the 5′-FSBA- and the 5′-FSBA- and DTT-treated enzyme preparations only, indicating that the labeled cysteine is not actually in the NAD+ binding site but in the near vicinity. Comparison of the amount of radiolabel incorporated into the peptide in the different preparations shows a dramatic increase has occurred in the 5′-FSBA- and DTT-treated enzyme over the 5′-FSBA-treated enzyme, indicating that the cysteine in this peptide is also involved in the disulfide bond. This peptide corresponds to residues 18–26 of the bovine enzyme, with the cysteine at position 20. In the human placental enzyme this residue has been substituted for an arginine.

Of the radiolabeled peptides, ^{274}EWGFCLDSR^{282} and ^{18}IICLLVEEK^{26} both exhibited a dramatic increase in the amount of ^{14}C incorporation in the 5′-FSBA- and DTT-treated enzyme compared to the 5′-FSBA-treated preparation. Both peptides exhibited an approximately equal increase (15-fold) in incorporation, suggesting that the cysteine residues in these two peptides are involved in the formation of the intramolecular disulfide bond that results in the inactivation of 3β-hydroxysteroid dehydrogenase activity upon incubation with 5′-FSBA.

None of the labeled peptides isolated in this study contain the so-called "glycine-rich" sequence, which has been found to be associated with the nucleotide binding sites of many dehydrogenase, kinases, and oxidoreductases. However, the peptide ^{18}IICLLVEEK^{26} is adjacent to the glycine-rich region of 3β-hydroxysteroid dehydrogenase, which we have proposed as being part of the NAD+ binding site (Rutherfurd et al., 1991). Of the five cysteine residues that were labeled in this study, it is intriguing to note that in the comparison with the human placental enzyme only one of these cysteines (residue 72) is unsubstituted.

It is interesting to note that both rat testicular (Ishii-Ohba et al., 1986b) and rat adrenal (Ishii-Ohba et al., 1986a) 3β-hydroxysteroid dehydrogenase activities are inhibited following treatment with 5′-FSBA. As with the bovine adrenal 3β-

hydroxysteroid dehydrogenase activity, the inhibition by 5′-FSBA could be protected against by the inclusion of NAD^+. Both the rat adrenal and testicular enzymes could be affinity-labeled by using [^{14}C]FSBA, which is in contrast to the bovine adrenal enzyme, which despite the use of several different methods could not be isolated with the [^{14}C]FSBA bound.

In their work on rat adrenal 3β-hydroxysteroid dehydrogenase/steroid isomerase, Ishii-Ohba et al. (1986a) reported that residues other than cysteine were likely to be involved in the inactivation of 3β-hydroxysteroid dehydrogenase activity by 5′-FSBA. The addition of DTT following inactivation by 5′-FSBA did not result in the reactivation of the rat adrenal enzyme; this is in contrast to the results of this study, where the addition of DTT resulted in the rapid and total regain of bovine adrenal 3β-hydroxysteroid dehydrogenase activity. This alone implies the importance of one or more cysteine residues at or in the near vicinity of the NAD^+ binding site in the bovine adrenal enzyme. In support of this, Hiwatashi et al. (1985), using bovine adrenal 3β-hydroxysteroid dehydrogenase, showed that *p*-chloromercuribenzoate, a highly specific sulfhydryl-modifying reagent (Fraenkel-Conrat, 1957), also inhibited the enzyme. This inhibition could be reversed by the addition of reducd glutathione and could be protected against by the presence of NADH, leading the authors to suggest that a cysteinyl residue may be an essential part of the NAD^+ binding site. It is possible that the difference in these results reflect variations in the nature of certain residues found at the NAD^+ binding site in the different species.

Although our studies do not indicate an essential role for cysteine residues in the catalytic activity of the enzyme, they do indicate the presence of certain cysteine residues around the NAD^+ binding site (residues that can be substituted by other amino acids without loss of NAD^+ binding) and show that cross-linking of two of these cysteine residues (20 and 278) can completely inactivate the enzyme. The identity of these cysteines will have importance in placing the NAD^+ binding site in a three-dimensional structure. It can be concluded that cysteines 20 and 278 are extremely close in the three-dimensional structure and that their cross-linking, catalyzed by 5′-FSBA, prevents NAD^+ from entering the binding site.

ACKNOWLEDGMENTS

We thank Mr. Michael Ronk for sequencing the radiolabeled peptides and Dr. Jerome Bailey for invaluable discussions on this work.

Registry No. 5′-FSBA, 57454-44-1; Cys, 52-90-4; 3β-hydroxysteroid dehydrogenase, 9015-81-0.

REFERENCES

Annamalai, A. E., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 10276–10283.
Brandt, M., & Levy, M. A. (1989) *Biochemistry 28*, 140–148.
Chen, S., & Guillory, R. J. (1981) *J. Biol. Chem. 256*, 4318–4323.
Chen, S., Haniu, M., Iyanagi, T., & Shively, J. E. (1986) *J. Protein Chem. 5*, 133–145.
Colman, R. F. (1983) *Annu. Rev. Biochem. 52*, 67–92.
Colman, R. F., Pal, P. K., & Wyatt, J. L. (1977) *Methods Enzymol. 46*, 240–248.
DePauw, E., Pelzer, G., Dang, D. V., & Marien, J. (1984) *Biochem. Biophys. Res. Commun. 123*, 27–32.
Esch, F. S., & Allison, W. S. (1978) *J. Biol. Chem. 253*, 6100–6106.
Fraenkel-Conrat, H. (1957) *Methods Enzymol. 4*, 247–269.
Gomi, T., Ogawa, H., & Fujioka, M. (1986) *J. Biol. Chem. 261*, 13422–13425.
Hawke, D. H., Harris, D. C., & Shively, J. E. (1985) *Anal. Biochem. 147*, 315–330.
Hiwatashi, A., Hamamoto, I., & Ichikawa, Y. (1985) *J. Biochem. (Tokyo) 98*, 1519–1526.
Ishii-Ohba, H., Saiki, N., Inano, H., & Tamaoki, B.-I. (1986a) *J. Steroid Biochem. 24*, 753–760.
Ishii-Ohba, H., Inano, H., & Tamaoki, B.-I. (1986b) *J. Steroid Biochem. 25*, 555–560.
Ishii-Ohba, H., Inano, H., & Tamaoki, B.-I. (1987) *J. Steroid Biochem. 27*, 775–779.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Liu, X.-F., Yuan, H., Haniu, M., Iyangi, T., Shively, J. E., & Chen, S. (1989) *Mol. Pharmacol. 35*, 818–822.
Luu-The, V., Lachance, Y., Labrie, C., Leblanc, G., Thomas, J. L., Strickler, R. C., & Labrie, F. (1989) *Mol. Endocrinol. 3*, 1310–1312.
Ogilvie, J. W. (1983) *Biochemistry 22*, 5915–5921.
Parsons, T. F., Buckman, J. D., Pearson, D. E., & Field, L. (1965) *J. Am. Chem. Soc. 30*, 1923–1926.
Rutherfurd, K. J., Chen, S., & Shively, J. E. (1991) *Biochemistry* (preceding paper in this issue).
Saradambal, K. V., Bednar, R. A., & Colman, R. F. (1981) *J. Biol. Chem. 256*, 11866-11872.
Takata, Y., & Fujioka, M. (1984) *Biochemistry 23*, 4357–4362.
Thomas, J. L., Berko, E. A., Faustino, A., Myers, R. P., & Strickler, R. C. (1988) *J. Steroid Biochem. 31*, 785–793.
Togashi, C. T., & Reisler, E. (1982) *J. Biol. Chem. 257*, 10112–10118.
Tomich, J. M., Marti, C., & Colman, R. F. (1981) *Biochemistry 20*, 6711–6720.
Witten, J. L., Schaffer, M. H., O'Shea, M., Cook, J. C., Hemling, M. E., & Rinehart, K. L. (1984) *Biochem. Biophys. Res. Commun. 124*, 350–358.
Zhao, H.-F., Simard, J., Labrie, C., Breton, N., Rhêaume, E., Luu-The, V., & Labrie, F. (1989) *FEBS Lett. 259*, 153–157.