Mitochondrial Nicotinamide Nucleotide Transhydrogenase: Active Site Modification by 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine†

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ABSTRACT: Membrane-bound and purified mitochondrial energy-linked nicotinamide nucleotide transhydrogenase (TH) was inhibited by incubation with 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), which is an analogue of TH substrates and their competitive inhibitors, namely, 5′-, 2′-, or 3′-AMP. NAD(H) and analogues, NADP, 5′-AMP, 5′-ADP, and 2′-AMP/3′-AMP mixed isomers protected TH against inhibition by FSBA, but NADPH accelerated the inhibition rate. In the absence of protective ligands or in the presence of NADP, FSBA appeared to modify the NAD(H) binding site of TH, because, unlike unmodified TH, the enzyme modified by FSBA under these conditions did not bind to an NAD-affinity column (NAD–agarose). However, when the NAD(H) binding site of TH was protected in the presence of 5′-AMP or NAD, then FSBA modification resulted in an inhibited enzyme that did bind to NAD–agarose, suggesting FSBA modification of the NADP(H) binding site or an essential residue outside the active site. [^{3}H]FSBA was covalently bound to TH, and complete inhibition corresponded to the binding of about 0.5 mol of [^{3}H]FSBA/mol of TH. Since purified TH is known to be dimeric in the isolated state, this binding stoichiometry suggests half-of-the-sites reactivity. A similar binding stoichiometry was found earlier for complete inhibition of TH by [^{14}C]DCCD [Phelps, D. C., & Hatefi, Y. (1984) *Biochemistry 23*, 4475–4480]. The active site directed labeling of TH by radioactive FSBA should allow isolation of appropriate peptides for sequence analysis of the NAD(H) and possibly the NADP(H) binding domains.

The mitochondrial energy-linked nicotinamide nucleotide transhydrogenase (TH; EC 1.6.1.1)[1] is a dimeric protein with a monomer molecular weight of (1.1–1.2) × 10^4 (Anderson & Fisher, 1981; Wu & Fisher, 1983). The enzyme catalyzes hydride ion transfer between NAD(H) and NADP(H) in a reaction that is coupled to transmembrane proton translocation, and exhibits half-of-the-sites reactivity (Phelps & Hatefi, 1984a). TH possesses two distinct binding sites for NAD(H) and NADP(H) (Rydstrom et al., 1976). The use of protein residue modifiers has revealed the possible presence in TH of arginyl (Djavadi-Ohaniance & Hatefi, 1975), cysteinyl (Earle et al., 1978; Wu & Fisher, 1982), lysyl (M. Yamaguchi and Y. Hatefi, unpublished results), and carboxyl (Phelps & Hatefi, 1981, 1984b; Pennington & Fisher, 1981) groups whose modifications result in enzyme inactivation and are prevented by the presence of one or both substrates. Among these, a putative carboxyl group modified by DCCD[1] appears to be at or near the NAD(H) binding site of TH, because (a) NAD(H) and analogues (5′-AMP and 5′-ADP), but not NADP(H) and analogues (2′- and 3′-AMP), protect TH against modification by DCCD and (b) unlike the unmodified TH, the DCCD-treated enzyme does not bind to NAD–agarose (Phelps & Hatefi, 1984a).

The present paper is concerned with the active site modification of TH by FSBA. This compound is an ATP analogue and has been used for covalent modification of the active site of F_1-ATPase from bovine heart mitochondria [Esch & Allison, 1978; see also Coleman et al. (1977)]. The studies with TH have suggested that radiolabeled FSBA might be a useful reagent for site-directed modification of this enzyme at either the NAD(H) or the NADP(H) binding domain.

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Materials and Methods

Materials. The sources of 5′-AMP, 5′-ADP, 2′-AMP/3′-AMP mixed isomers, NAD(H), NADP(H), AcPyAD(H), NAD–agarose, and Brij 58 were the same as before (Phelps & Hatefi, 1984a). FSBA was from Sigma, dimethyl sulfoxide from Baker, and 3-(*N*-morpholino)propanesulfonic acid (MOPS; Ultrol brand) from Calbiochem. [^{3}H]FSBA (19.6 × 10^3 cpm/nmol), tritiated primarily at the C-2 hydrogens of the adenine moiety, was the generous gift of Dr. W. S. Allison, University of California, San Diego. Other chemicals used were reagent grade or of the highest quality available.

SMP was prepared from bovine heart mitochondria according to the procedure of Löw & Vallin (1963), and TH was purified from SMP essentially according to Wu et al. (1982) as detailed elsewhere (Phelps & Hatefi, 1984a). The specific activity of the purified TH preparations used in these studies varied between 10 and 15 μmol of AcPyAD reduced by NADPH min^{-1} (mg of protein)$^{-1}$.

Assays. Protein was determined by the method of Lowry et al. (1951), and the transhydrogenase activities of SMP (Phelps & Hatefi, 1981) and the purified TH (Phelps & Hatefi, 1984a) were assayed as described with NADPH and AcPyAD as hydride ion donor and acceptor, respectively.

Modification of SMP or TH with FSBA. Unless otherwise indicated, SMP at 5 mg/mL in 0.25 M sucrose containing 50 mM Tris–sulfate, pH 7.5, or TH at 0.2–0.35 mg/mL in 5 mM sodium phosphate, pH 7.5, containing 0.01% Brij 58 was in-

[1] Abbreviations: AcPyAD and AcPyADH, oxidized and reduced 3-acetylpyridine adenine dinucleotide, respectively; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; MOPS, 3-(*N*-morpholino)propanesulfonic acid; SMP, bovine heart submitochondrial particles; TH, purified nicotinamide nucleotide transhydrogenase; ATPase, adenosinetriphosphatase; Tris, tris(hydroxymethyl)aminomethane.

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cubated at room temperature with the indicated amount of FSBA or [^{3}H]FSBA in anhydrous dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the incubation mixtures, and in controls lacking FSBA, was ≤1.4%. Where indicated, nucleotides were added to the incubation mixtures prior to FSBA addition. Because of the high pH sensitivity of TH modification by FSBA, care was exercised to adjust the pH of the incubation mixtures to pH 7.5 before FSBA was added. For inhibition studies, 10-μL aliquots of the SMP or the TH incubation mixtures were removed at the indicated intervals and assayed for transhydrogenase activity as described.

In experiments where the binding of FSBA-modified TH to NAD–agarose was studied, 0.6–0.7-mL aliquots of the TH incubation mixtures were passed through two consecutive 3-mL Sephadex G-25 (medium) centrifuge columns (Penefsky, 1977), preequilibrated with the incubation buffer, in order to remove the added nucleotides and unreacted FSBA. Then, the filtered TH was placed on NAD–agarose and eluted as described elsewhere (Phelps & Hatefi, 1984a), except that the buffer wash following the NADH-containing buffer was increased to 6 mL. It was found that, after elution of the active TH band, subsequent washing of the column with 2 M NaCl resulted in elution of up to 20% of the protein applied to the column. This fraction had only a marginal transhydrogenase activity, its occurrence was not related to the prior treatment of TH with FSBA ± nucleotides, and it had the same electrophoretic mobility on dodecyl sulfate–acrylamide gels as TH. Elsewhere (Phelps & Hatefi, 1984), we had shown that rechromatography of TH purified and stored at 0 °C on NAD–agarose increased the specific activity of the eluted TH band. It seems possible that upon standing purified TH undergoes a slow denaturation or aggregation and that a second chromatography on NAD–agarose can separate active TH from the partially denatured or aggregated enzyme.

In studies concerned with the labeling of TH with [^{3}H]-FSBA, 0.2-mL aliquots of the TH incubation mixture containing [^{3}H]FSBA ± nucleotides were removed at various time intervals and immediately filtered through two successive 1-mL Sephadex-centrifuge columns as above. The filtrates were stored on ice until used. At the same time points, 10-μL aliquots of the TH incubation mixtures were also removed and assayed for transhydrogenase activity. For determination of TH-bound radioactivity, the above filtrates were each passed through a third Sephadex-centrifuge column. Then, 80-μL aliquots were boiled for 2 min in the presence of 1% sodium dodecyl sulfate and subjected to electrophoresis on 6-cm cylindrical polyacrylamide gels according to the procedure of Laemmli (1970) as described elsewhere (Phelps & Hatefi, 1984a). The electrophoresis was performed, however, at 100 V, instead of 150 V, to minimize heating, and fixing, staining, and destaining were deleted, because of the lability of FSBA to acidic or basic conditions. Thus, a control gel was fixed, stained with Coomassie blue, and destained to localize the position of TH, and the other unstained gels were frozen on a block of dry ice and stored at −70 °C. Guided by the position of TH on the stained gel, 1 cm around the corresponding position of each unstained gel was cut in 2-mm slices, and each slice was digested and its radioactivity determined as before (Phelps & Hatefi, 1984a). The radioactivity of the slice with the highest cpm plus the radioactivity of one slice on each side (i.e., the radioactivity of 6 mm of the gel) was taken as the bound radioactivity of each TH sample. The remaining sections of the gels had a uniform background radioactivity, which was the same as that of [^{3}H]FSBA incubated in the absence of TH and subjected to electrophoresis under identical conditions as above. This background radioactivity was subtracted from the total radioactivity of the 6 mm of the gels containing TH-bound [^{3}H]FSBA.

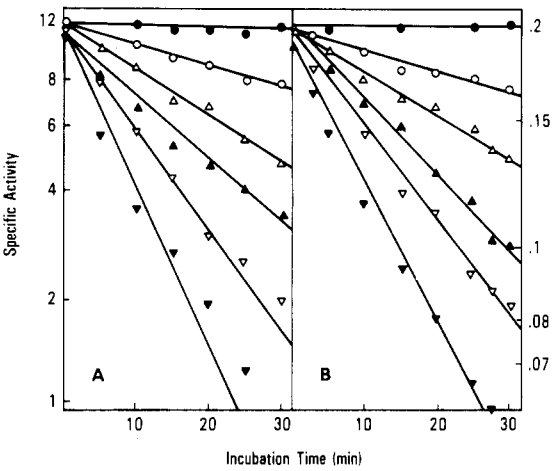

FIGURE 1: Semilogarithmic plots of the inhibition time course of the NADPH → AcPyAD transhydrogenase activities of purified TH (A) and SMP (B) incubated with various concentrations of FSBA. The concentration of purified TH in the incubation mixture was 0.27 mg/mL, and the FSBA concentrations were zero (●), 0.1 (○), 0.3 (△), 0.5 (▲), 0.8 (▽), and 2.0 (▼) mM. Other conditions were the same as described under Materials and Methods.

Table I: Effects of Substrates and Analogues on Inhibition of TH by FSBA[a]

additions	$k - k_0$ (min^{-1}) SMP	TH
none	0.029	0.062
AcPyAD, 2.0 mM	0.011	0.020
AcPyADH, 2.0 mM	0.003	0.023
NADP, 2.0 mM	0.014	0.030
NADPH, 2.0 mM	0.054	0.133
5′-AMP, 4.0 mM	0.007	0.013
5′-ADP, 4.0 mM	0.003	0.008
2′- + 3′-AMP, 6.3 mM	0.008	0.009

[a] SMP (5 mg/mL in 0.25 M sucrose containing 50 mM Tris–sulfate, pH 7.5) or TH (0.25–0.35 mg/mL in 5 mM sodium phosphate, pH 7.5, containing 0.01% Brij 58 and 1.4% dimethyl sulfoxide) was incubated at 23 °C in the presence of 1.0 mM FSBA and the substrates or analogues as indicated. Duplicate experiments were carried out in the absence of FSBA. At regular time intervals, 10-μL aliquots were withdrawn and assayed as described under Materials and Methods. k and k_0, pseudo-first-order inhibition rate constants in the presence and absence of FSBA, respectively.

RESULTS

As mentioned earlier, FSBA has been used as an analogue of ATP to label the ATP binding sites of the mitochondrial F_1-ATPase (Esch & Allison, 1978). Since in TH 5′-AMP and 5′-ADP are competitive inhibitors of NAD(H) (Rydström et al., 1976), we reasoned that FSBA might be useful as an affinity reagent for labeling the NAD(H) binding site of this enzyme. As shown in Figure 1, FSBA inhibited the transhydrogenase activities of both SMP and the purified TH. The inhibitions were pseudo first order at the FSBA concentration range and the incubation time shown in Figure 1. Longer incubations resulted in a progressive decrease in the slope of the semilogarithmic plots, which could be related to the known instability of FSBA in aqueous media (Esch & Allison, 1978). Various substrates and analogues, except NADPH, protected the transhydrogenase activities of both SMP and the purified preparation against the inhibitory effect of FSBA (Table I). NADPH caused a 2-fold increase in the rate of inhibition,

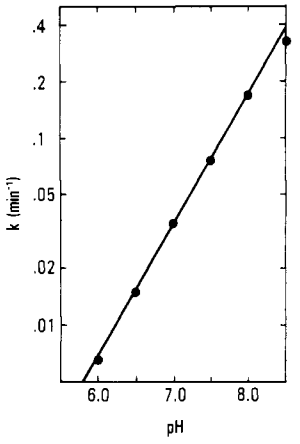

FIGURE 2: Effect of pH on the rate of inhibition of the transhydrogenase activity of SMP by FSBA. SMP at 2.5 mg/mL was suspended in a buffer made up by mixing 0.25 M sucrose containing 25 mM Tris with 0.25 M sucrose containing 25 mM MOPS to obtain the desired pH. Then 1 mM FSBA was added, and the mixture was incubated and sampled for assay of transhydrogenase activity as described under Materials and Methods and Figure 1. k, pseudo-first-order inhibition rate constant at the pH values indicated.

which was reminiscent of the stimulatory effect of NADPH on inhibition of TH by DCCD (Phelps & Hatefi, 1984a,b) or *N*-ethylmaleimide (Earle et al., 1978). The finding that AcPyAD(H), 5′-AMP, 5′-ADP, NADP, or the 2′- and 3′-AMP mixed isomer protected TH against inhibition by FSBA suggested that the residue modified by FSBA is either at the TH active site or in a location that undergoes substrate-induced conformation change and thereby alters the accessibility of the FSBA target. Figure 2 shows the effect of pH on the inhibition of TH by FSBA. As seen in this figure, the pseudo-first-order inhibition rate constant increased 50-fold when the pH of the incubation mixture was increased from pH 6.0 to pH 8.5. Further, the semilogarithmic plot of the pseudo-first-order inhibition rate constant vs. pH was a straight line in the pH range 6.0–8.5. These results suggest participation of a basic group in TH inactivation with $pK_a > 8.5$. In bovine heart F_1-ATPase, where FSBA reacts with an active site tyrosyl residue, it was also shown that the rate of F_1-ATPase inactivation increased severalfold in going from pH 6.5 to pH 8.0 (Esch & Allison, 1978).

In order to better characterize the TH region modified by FSBA, the FSBA-inhibited enzyme was subjected to affinity chromatography on NAD–agarose. It was shown elsewhere (Phelps & Hatefi, 1984a) that unmodified TH binds to NAD–agarose and can be eluted by NADH. However, when FSBA-modified TH was placed on an NAD–agarose column, the inhibited enzyme did not bind and was washed through with buffer alone (Figure 3A). These results indicated that FSBA had modified TH in such a manner that the modification interfered with its binding to NAD–agarose. Essentially similar results were obtained when the FSBA modification of TH was carried out in the presence of NADP, presumably to protect the NADP-binding site from attack by FSBA. However, when TH in the presence of 5′-AMP to protect the NAD(H)-binding site was incubated with FSBA, the enzyme after inhibition did bind to NAD–agarose (Figure 3B). Similar

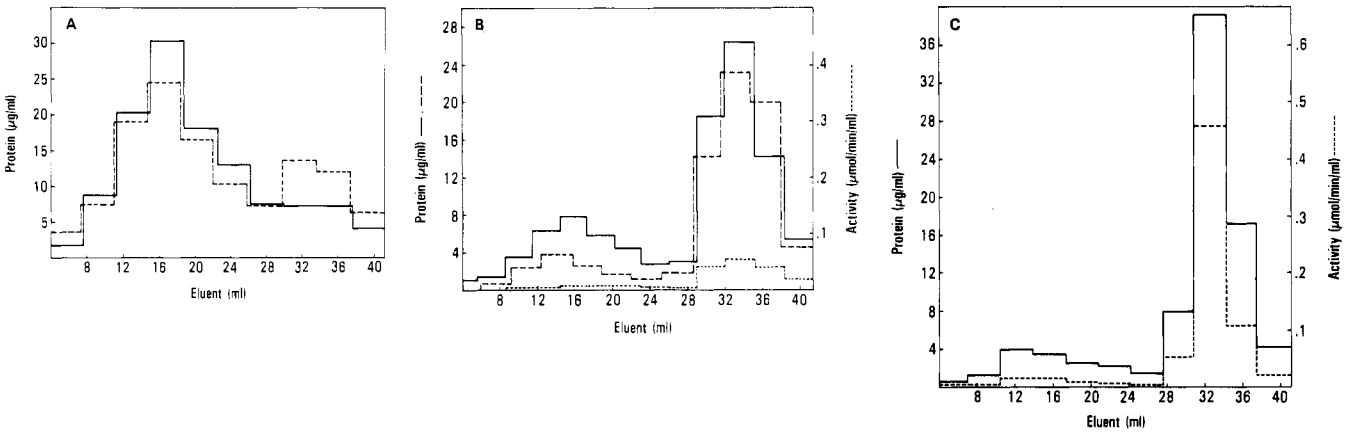

FIGURE 3: Affinity chromatography on NAD–agarose of purified TH (A) treated with FSBA, (B) treated with FSBA in the presence of 5′-AMP, and (C) untreated. (A) TH at 0.32 mg/mL was treated with 1.5 mM FSBA. After 35 min, at which time TH was 90% inhibited, half of the mixture was removed and filtered through Sephadex-centrifuge columns in 0.5-mL aliquots. The remainder was incubated for an additional 85 min, at which time TH activity was no longer detectable, and then passed through Sephadex-centrifuge columns as before. The pHs of the effluents were adjusted to pH 6.8 with the addition of 10 mM NaH_2PO_4 before applying them to 1.0 × 10 cm NAD–agarose columns at a rate of 0.5 mL/min. The columns were eluted with the following sequence of buffers at a rate of 0.5 mL/min: 4 mL of column buffer [see Phelps & Hatefi (1984a)], pH 7.0, containing 0.1 mM NADP; 12 mL of column buffer, pH 7.0, containing 20 mM NaCl; 4 mL of column buffer, pH 7.0; 7 mL of column buffer, pH 7.5, containing 0.55 mM NADH; 6 mL of column buffer, pH 7.0; 8 mL of column buffer, pH 7.0, containing 2 M NaCl. The points at which various elution buffers were experienced coming off the columns were buffer containing NADP at 12.5–13.0 mL, buffer containing 20 mM NaCl at 16.5–17.0 mL, buffer containing NADH at 32.5–33 mL, and buffer containing 2 M NaCl at 45.5–46 mL (not shown). (Dashed line) TH incubated with FSBA for 35 min (90% inhibited); (solid line) TH incubated with FSBA for 120 min (completely inhibited). (B) (Solid line) TH (0.23 mg/mL) in the presence of 4.0 mM 5′-AMP was treated with 0.6 mM FSBA in 0.12% dimethyl sulfoxide. After 60 and 120 min of incubation, further additions each of 0.6 mM FSBA were made, and the incubation was continued for a total of 210 min. At this point, the FSBA-treated TH exhibited 5% of its original activity, and the control treated only with 5′-AMP and dimethyl sulfoxide exhibited 56% of its original activity. (Dashed line) TH (0.2 mg/mL) in the presence of 4.0 mM 5′-AMP was treated with 1 mM FSBA in 0.7% final concentration of dimethyl sulfoxide. The incubation was continued for 270 min at which point 83% of the transhydrogenase activity was lost. The incubation mixtures were then filtered through Sephadex-centrifuge columns, placed on NAD–agarose columns, and eluted as before. The points at which various elution buffers were experienced coming off the NAD–agarose columns were buffer containing NADP at 10–10.5 mL, buffer containing 20 mM NaCl at 14–14.5 mL, buffer containing NADH at 30–30.5 mL, and buffer containing 2 M NaCl at 43–43.5 mL (not shown). (Dotted line) Transhydrogenase activity associated with the protein elution profile shown as a solid line. (C) TH in the presence of 4.0 mM 5′-AMP treated as in the first experiment of panel B (solid line), except that the additions made were only of dimethyl sulfoxide in the absence of FSBA. The points at which the elution buffers were experienced coming off the NAD–agarose column were buffer containing NADP at 10.5 mL, buffer containing 20 mM NaCl at 14.5 mL, buffer containing NADH at 30.5 mL, and buffer containing 2 M NaCl at 37.5 mL (not shown). Others conditions were the same as described under Materials and Methods.

results were obtained when TH in the presence of NAD was incubated with FSBA, except that, in agreement with the data of Table I, the protection of the NAD(H) site by NAD was not as strong as that shown in Figure 3B (data not shown). Figure 3C shows the elution pattern of unmodified TH chromatographed on NAD–agarose. Other controls in which TH in the absence of FSBA was incubated with 5′-AMP, NAD, or NADP, then freed of the added nucleotide by filtration through Sephadex-centrifuge columns, and placed on NAD–agarose showed the same binding characteristics as that depicted in Figure 3C. The above results indicate the following. (i) Inhibition of TH by covalent protein modification does not necessarily abolish the ability of the enzyme to bind to NAD–agarose in a manner that it can be eluted by NADH. (ii) Treatment of TH with FSBA in the absence of ligands to protect the NAD(H)-binding site of the enzyme probably results in modification of this site. This is consistent with the fact that such ligands protect TH against inhibition by FSBA and with the finding that FSBA modification of unprotected TH results in an inhibited enzyme which does not bind to NAD–agarose. (iii) TH in the presence of 5′-AMP to protect the NAD(H)-binding site can still be inhibited, albeit slowly, by FSBA. The enzyme so inhibited binds to NAD–agarose, suggesting that under these conditions FSBA modification does not involve the NAD(H)-binding site, but could be occurring at the NADP(H)-binding site. These considerations allowed the possibility that in the absence of any protective agent both the NAD(H)-binding and the NADP(H)-binding sites of the same enzyme molecule might be modified by FSBA. However, as will be seen below, such a double modification of TH by FSBA does not appear to take place.

When [^{14}C]DCCD was used, it was shown elsewhere (Phelps & Hatefi, 1984a) that the dimeric TH exhibits half-of-the-sites reactivity and that complete inhibition corresponds to the covalent binding of 1 mol of [^{14}C]DCCD per TH dimer. Similar results were obtained in the present study with [^{3}H]FSBA. As seen in Figure 4, data in the absence or presence of various protective ligands all extrapolated at 100% activity inhibition to the binding of about 0.5 mol of [^{3}H]-FSBA/mol of TH. Figure 4 also shows that protection of TH activity partially by NADP or to a greater extent by 5′-AMP or 5′-AMP plus NADP also resulted in a corresponding decrease in the labeling of TH by [^{3}H]FSBA. Thus, during the same incubation time that TH alone was 83% inhibited and took up 0.42 mol of [^{3}H]FSBA/mol, the corresponding values for TH + NADP, TH + 5′-AMP, and TH + NADP + 5′-AMP were respectively 70% inhibition at 0.32 mol of [^{3}H]-FSBA bound/mol, 40% inhibition at 0.2 mol of [^{3}H]FSBA bound/mol, and 32% inhibition at 0.17 mol of [^{3}H]FSBA bound/mol. As seen in Figure 4, the line drawn through the data points intersects the ordinate above the origin, suggesting some noninhibitory adherence of [^{3}H]FSBA to TH. However, the fact that the data points for TH in the absence or presence of different protective ligands are all located on or near this line indicates that in all cases the degree of binding achieved was associated with modification of equally essential sites. Further, the extrapolation at 100% activity inhibition to ~0.5 mol of [^{3}H]FSBA bound/mol of TH indicates that only one essential site was modified per TH dimer, regardless of the nature or the presence of protective ligands. These conclusions and the data of Figure 3 are, therefore, consistent with the possibility that FSBA modification occurs preferentially at the NAD(H)-binding site, but when this site is protected with appropriate ligands, FSBA can be directed to modify the NADP(H)-binding site.

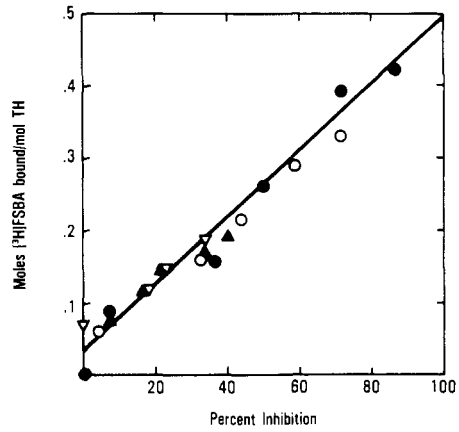

FIGURE 4: Binding of [^{3}H]FSBA to purified TH and inhibition of transhydrogenase activity in the absence and presence of protective ligands. TH at 0.23 mg/mL in the absence (●) or presence of 4.0 mM NADP (○), 7.1 mM 5′-AMP (▲), or 4.0 mM NADP plus 7.1 mM 5′-AMP (▽) was incubated for a total of 65 min with 0.98 mM [^{3}H]FSBA. Control samples of TH were also incubated under identical conditions in the absence of [^{3}H]FSBA. At regular intervals, samples were withdrawn and assayed for transhydrogenase activity. Also, at the inhibition points shown, samples were removed and analyzed for the extent of bound radioactivity. Assays for transhydrogenase activity and TH-bound radioactivity are described under Materials and Methods. The pseudo-first-order inhibition rate constants (min^{-1}) calculated for TH in the absence and presence of protective ligands were the following: TH + FSBA, 0.039; TH + NADP + FSBA, 0.014; TH + 5′-AMP + FSBA, 0.007; TH + NADP + 5′-AMP + FSBA, 0.005.

DISCUSSION

It has been shown that FSBA, which is a structural analogue of ATP (Esch & Allison, 1978), inhibits the membrane-bound and purified TH and that the enyzme can be protected against this inhibition by NAD(H) and its compettive inhibitors 5′-AMP and 5′-ADP, or by NADP and its competitive inhibitors, 2′- plus 3′-AMP. The degree of inhibition in the absence or presence of different protective ligands is correlated with the covalent binding of [^{3}H]FSBA to purified TH, and complete inhibition corresponds to the binding of approximately 0.5 mol of [^{3}H]FSBA/mol of TH. Since TH is known to be dimeric both in the intact mitochondrial inner membrane and in the purified state (Anderson & Fisher, 1981; Wu & Fisher, 1983), this binding stoichiometry suggests half-of-the-sites reactivity or site–site "anticooperativity" with respect to ligand binding. A similar binding stoichiometry was reported earlier (Phelps & Hatefi, 1984a) for the inhibition of TH by covalent modification with [^{14}C]DCCD, which appeared to occur at the NAD(H) binding site of the enzyme. Whether by analogy to F_1-ATPase or cytochrome *c* oxidase the apparent half-of-the-sites reactivity or site–site anticooperativity of TH should be considered of mechanistic sigificance with regard to energy transduction by this enzyme remains to be seen. The mitochondrial ATPase, which contains three catalytic sites per molecule, exhibits negative cooperativity with respect to [ATP] (Ebel & Lardy, 1975; Wong et al., 1984), and positive catalytic cooperativity in the sense that product removal from one site (a rate-determining step) is greatly facilitated by substrate binding to additional sites (Grubmeyer et al., 1982; Cross et al., 1982; Gresser et al., 1982), and these characteristics appear to be fundamental features of the mechanism of ATP synthesis by this enzyme (Boyer, 1979; Gresser et al., 1982; O'Neal & Boyer, 1984). In bovine cytochrome *c* oxidase, it has been shown that covalent binding of 1 mol of derivatized cytochrome *c* per cytochrome oxidase dimer completely blocks electron transfer to the enzyme from added ferrocytochrome *c* [for reviews, see Wikström et al. (1981) and Capaldi et al. (1983)],

and this apparent half-of-the-sites reactivity or site–site anticooperativity has been considered by Wikström et al. (1981) as a clue to the mechanism of energy transduction by cytochrome oxidase.

When added to TH in the absence of protective ligands, FSBA appears to bind preferentially at or near the NAD(H)-binding site of the enzyme. This conclusion is supported by the facts that (a) FSBA inhibition is prevented by NAD(H) and its competitive inhibitors, (b) the FSBA-modified enzyme does not bind to NAD–agarose, and (c) when the NAD(H)-binding site is protected from attack by appropriate ligands, FSBA binds elsewhere and inhibits TH, but the inhibited enzyme now binds to NAD–agarose in a manner similar to that of unmodified TH. Whether TH contains regulatory nucleotide-binding sites and, under conditions that the NAD(H)-binding site is protected, FSBA binds to these regulatory sites and inhibits the enzyme is not known. However, the combined results of Figures 3 and 4, as discussed in the preceding section, suggest that when the NAD(H)-binding site is protected, FSBA inhibits TH by modifying the NADP(H)-binding site. We have shown recently (M. Yamaguchi and Y. Hatefi, unpublished results) that pyridoxal phosphate inhibits TH and that the enzyme can be protected against this inhibition by NADP(H), but not by NAD(H). Since the inhibition by pyridoxal phosphate can be reversed by L-lysine, it should be interesting to see whether the pyridoxal-modified TH in the presence of 5′-AMP would offer severe resistance to modification by FSBA.

In F_1-ATPase, the segment around the FSBA-modified tyrosyl residue of the β-subunit appears to have a highly conserved structure as evidenced by the sequence homology of the corresponding segments of the bovine and the *Escherichia coli* enzymes (Saraste et al., 1981). Work is currently in progress to use radioactive FSBA for active site directed affinity labeling and isolation of oligopeptides corresponding to the NAD(H)- and the NADP(H)-binding domains of TH. It should be interesting to compare the primary structures of these oligopeptides and to see whether there are features in common with the region in F_1-ATPase that binds FSBA.

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Registry No. TH, 9014-18-0; FSBA, 57454-44-1; AcPyAD, 86-08-8; AcPyADH, 153-59-3; NADP, 53-59-8; 5′-AMP, 61-19-8; 5′-ADP, 58-64-0; 2′-AMP, 130-49-4; 3′-AMP, 84-21-9; NAD, 53-84-9; NADH, 58-68-4.

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