Structure–Function Relationship of NAD(P)H:Quinone Reductase: Characterization of NH_2-Terminal Blocking Group and Essential Tyrosine and Lysine Residues†

Mitsuru Haniu,*,‡ Henry Yuan,‡ Shiuan Chen,‡ Takashi Iyanagi,§ Terry D. Lee,‡ and John E. Shively‡

Division of Immunology, Beckman Research Institute of the City of Hope, Duarte, California 91010, and Division of Biochemistry, Institute of Basic Medical Sciences, The University of Tsukuba, Niihari-gun, Ibaraki-ken, 305, Japan

Received June 9, 1987; Revised Manuscript Received March 15, 1988

ABSTRACT: The amino terminal blocked peptide of rat liver NAD(P)H:quinone reductase (DT-diaphorase) was determined by amino acid sequence analysis and by mass spectrometry. The mature protein is composed of 273 amino acids and contains an acetylated amino terminus, which was not identified by previous cDNA analysis. The enzyme was inactivated by *p*-nitrobenzenesulfonyl fluoride (NBSF) or 2,4,6-trinitrobenzenesulfonate (TNBS) with pseudo-first-order kinetics. These studies suggest that essential tyrosine and lysine may be present in the active site of this enzyme. The NBSF inhibition was protected by 1-naphthol and 1-naphthylamine, but not by NAD^+. However, TNBS inhibition was not prevented by the naphthalene derivatives or NAD^+. Specific peptides labeled with NBSF or TNBS were isolated by high-performance liquid chromatography and were sequenced. These analyses revealed that the NBSF-labeled tyrosine resides in a predominantly hydrophobic region and TNBS-labeled lysine in a predominantly hydrophilic region.

The flavoprotein NAD(P)H:quinone reductase (DT-diaphorase) promotes the two-electron reduction of many types of quinones including vitamin K (menadione) to hydroquinones by both NADH and NADPH (Ernster et al., 1960; Iyanagi & Yamazaki, 1970; Iyanagi, 1987). The enzyme can be inhibited by dicoumarol and a related vitamin K antagonist (Ernster et al., 1962; Hosoda et al., 1974; Hollander & Ernster, 1975). This enzyme plays important roles protecting against the toxicity of quinones, which occur widely in nature, including superoxide radicals and semiquinone free radicals (Thor et al., 1982; Lind et al., 1982; Miller et al., 1986). Physiologically, the liver enzyme is involved in the metabolism of vitamin K, which regulates the blood coagulation mechanism (Wallin et al., 1978). Brain NADPH-diaphorase is implicated in the pathology of Huntington's disease where the patient's brain tissue contains an abnormally high activity of the diaphorase (Ferrante et al., 1985). Neurons containing the diaphorase are selectively resistant to the endogenous toxic amino acids, quinolinate, or L-glutamate. Selective sparing of neurons containing this enzyme is a hallmark of Huntington's disease (Koh et al., 1986), but the relationship between this enzyme and the disease has not been fully elucidated.

DT-diaphorase is induced by polycyclic aromatic compounds such as 2(3)-*tert*-butyl-4-hydroxyanisole (BHA) and 3-methylcholanthrene (3-MC) and by azo dyes (Talalay & Benson, 1980). Molecular weight determination suggests that this enzyme consists of two identical subunits having a molecular weight of 30 000. Prochaska and Talalay (1986) reported two forms of the enzyme from mouse liver, which were designated "hydrophilic" and "hydrophobic" forms on the basis of the order of elution from phenyl-Sepharose. However, multiple forms of this enzyme have not been demonstrated in rat liver.

cDNA clones complementary to the mRNA for this enzyme have been constructed (Williams et al., 1986), and the cDNA sequence was recently determined by Robertson et al. (1986) and Bayney et al. (1987). The amino acid sequences deduced by these two groups differed from each other in the amino-terminal initiation. The amino acid sequence of this enzyme did not match any of the sequences of FAD binding proteins in the protein data bank (Robertson et al., 1986; Bayney et al., 1987). In this paper, we report the complete amino acid sequence of this enzyme, including an amino-terminal blocking group and the existence of functional resiudes including tyrosine and lysine residues.

Materials and Methods

Materials. Menadione and NADH were purchased from Sigma Chemical Co. *p*-Nitrobenzenesulfonyl fluoride (NBSF)[1] was obtained from Pierce Chemical Co. 2,4,6-Trinitrobenzenesulfonate (TNBS) was from Sigma. Carboxypeptidase Y and *Staphylococcus aureus* protease were from Pierce. TPCK–trypsin was purchased from Cooper Biomedical Co., and carboxypeptidase B was from Worthington Biochemicals. TFA, ethyl acetate, butyl chloride, and HFBA were sequencer grade as reported previously (Shively et al., 1982). HPLC-grade acetonitrile was obtained from J. T. Baker Chemical Co.

Purification of Enzyme. DT-diaphorase was purified from livers of female Wistar rats injected daily for 3 days with 3-MC (4 mg/100 g of body weight) essentially according to the method of Höjeberg et al. (1981). Livers from 50 rats were homogenized in 0.15 M KCl, and the homogenate (20% wet by volume) was centrifuged at 10000*g* for 20 min. The supernatant was centrifuged at 78000*g* for 2 h, and the super-

† This research was supported by National Institutes of Health Grant GM 34426 and National Cancer Institute Grant CA33572 to the City of Hope Cancer Center.

* To whom correspondence should be addressed.
‡ Beckman Research Institute of the City of Hope.
§ The University of Tsukuba, Niihari-gun.

[1] Abbreviations: DTE, dithioerythreitol; DTT, dithiothreitol; HFA, hexafluoroacetone trihydrate; HFBA, heptafluorobutyric acid; 3-MC, 3-methylcholanthrene; NEM, *N*-ethylmaleiimide; PITC, phenyl isothiocyanate; PTH, phenylthiohydantoin; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; TNBS, 2,4,6-trinitrobenzenesulfonate; TPCK, tosylphenylalanine chloromethyl ketone; FAB/MS, fast atom bombardment mass spectrometry; FD/MS, field desorption mass spectrometry; NBSF, *p*-nitrobenzenesulfonyl fluoride.

0006-2960/88/0427-6877$01.50/0

natant fraction was applied to an azodicoumarol–Sepharose 4B column (2 × 10 cm) equilibrated with 0.25 M sucrose in 50 mM Tris-HCl, pH 7.5 (buffer A). After application of the sample, the gel was washed with 2 M KCl in 0.25 M sucrose and 50 mM Tris-HCl, pH 8.9. The enzyme was eluted with 30 mL of 10 mM NADH in buffer A. The active fractions were dialyzed with 10 mM potassium phosphate buffer, pH 7.7 (buffer B), and applied to an hydroxylapatite column (1.5 × 10 cm) equilibrated with buffer B and washed with same buffer. The enzyme was eluted with 300 mM potassium phosphate buffer, pH 7.7, and active fractions were concentrated by ultrafiltration membrane cones (CF 25). The concentrated sample was applied to a Sephacryl S-200 column (3 × 80 cm) equilibrated with buffer A. The purified enzyme had a high activity for menadione as a substrate.

Peptide Preparation. Purified enzyme was carboxymethylated with radiolabeled [^{14}C]iodoacetate in 6 M guanidine hydrochloride buffer, pH 8.5. The sample was dialyzed against water until radioactivity was not detected in the dialysate (2 days) and then lyophilized. Proteolytic cleavages were performed by TPCK–trypsin, α-chymotrypsin, or *S. aureus* protease for 24 h in 0.2 M ammonium bicarbonate, pH 8.0, with enzyme protein ratios of 1:50 w/w. Cyanogen bromide cleavage was carried out in 70% formic acid for 24 h at room temperature.

Peptide Separation. Peptides were separated by HPLC using several columns such as Ultrasphere C8 (4.6 × 250 mm, 5 μm), C18 (4.6 × 250 mm, 5 μm), Vydac C4 (4.6 × 250 mm), and μBondapak phenyl (4.1 × 250 mm). Peptides were eluted with a linear gradient from solvent A (0.1% TFA in water) to solvent B (0.1/9.9/90 TFA/H_2O/CH_3CN, v/v/v) over 60 min with a flow rate of 1.0 mL/min. Peptides were detected at 220 and 350 nm for TNBS-peptides in pH 2 buffer.

Analytical Methods. Automated sequence analysis of the protein was performed by a gas-phase sequencer built by City of Hope (Hawke et al., 1985). All of the peptides were sequenced by a manual microsequencing strategy as described elsewhere (Haniu et al., 1986). Reaction volumes were minimized to 30 μL of coupling buffer and 20 μL of HFBA. The solvent for removal of excess PITC and extraction of PTH amino acids was butyl chloride instead of benzene. Phenylthiohydantoin derivatives were analyzed by reverse-phase HPLC as previously reported (Hawke et al., 1982). Amino acid compositions of the peptides were determined on a Beckman Model 121MB amino acid analyzer after acid hydrolysis in 6 N HCl containing 0.1% 2-mercaptoethanol for 24 h at 110 °C (Del Valle & Shively, 1979).

Inhibition Studies with NBSF. The DT-diaphorase (0.65 μM) was incubated with different concentrations of NBSF/ethanol solution (0–30 μM) in the absence of coenzyme or substrate at room temperature, as described previously (Liao et al., 1982). At each time interval, one aliquot (10 μL) was taken for enzyme assay, followed by addition of 10 μL of 8 mM NADH and 10 μL of 8 mM menadione in 1.0 mL of 0.1 M phosphate buffer, pH 7.4.

Effect of Naphthalene Derivatives on NBSF Inhibition. The enzyme was initially incubated with effectors such as 1-naphthol or 1-naphthylamine for 30 min at room temperature. The concentrations of the effectors were 0.2–0.4 mM in each case.

Modification of ϵ-Amino Groups. The enzyme was incubated in 0.1 M phosphate buffer, pH 7.4, with various concentrations of TNBS. TNBS concentrations were 0–50 μM. After incubation at room temperature, the enzyme activity was assayed. At each time interval, duplicate values were measured and enzyme controls were included. For experiments investigating the effect of NAD^+ or NADH on NBSF inhibition, enzyme incubation with 10 μL of 8 mM coenzyme was carried out at room temperature for 1 h prior to TNBS labeling.

Effect of the Naphthalene Derivatives on TNBS Inhibition. The enzyme was initially incubated in 0.1 M phosphate buffer, pH 7.4, with several naphthalene derivatives (0.4 mM) at room temperature. TNBS inhibition or remaining enzyme activity was measured as described above.

Stoichiometry of the Labeled Lysines with TNBS. The number of the lysine residues reacted with TNBS was measured according to the methods of Fields (1971). The enzyme (1 nmol) was incubated with 10–100 nmol of TNBS in phosphate buffer, pH 7.4, for 1 h at 25 °C in the presence or the absence of 2-fold molar excess of menadione or dicoumarol. Sodium sulfite (0.3 mM) was added to the reaction mixtures, and the product formation with the TNP-peptide was quantitated by measuring the absorbance at 420 nm. For the calculation of the number of TNP-lysine residues, an extinction coefficient of 19 200 M^{-1} cm^{-1} was used.

Isolation of the Labeled Peptides. For isolation of NBSF- or TNBS-labeled peptides, the enzyme (11 nmol) was incubated with NBSF or TNBS solution (100-fold molar excess) in 1.0 mL of 0.1 M phosphate buffer, pH 7.4, at room temperature. After 2 h, the reaction mixtures were applied to a Sephadex G-25 column (1 × 21 cm) equilibrated with 0.1 M phosphate buffer, pH 7.4, to remove the excess reagent. Simultaneously, one aliquot (10 μL) was taken for enzyme assay without passing through the gel column. After the absorption spectra were measured to determine the stoichiometry of TNBS labeling, the proteins were digested with TPCK–trypsin in 0.1 M ammonium bicarbonate, pH 8.0, for 24 h at 37 °C and applied to a Vydac C4 reverse-phase HPLC column (4.6 × 250 mm). The NBS-peptides were detected by two wavelength measurements at 254 and 280 nm. The TNP-peptides were detected by absorbance at 350 nm.

Mass Spectrometry. FAB mass spectra were taken with a JEOL HX100HF mass spectrometer operating at 5-kV accelerating potential and a nominal resolution setting of 3000. A neutral xenon atom beam with 3-kV translational energy was used for sample ionization. Peptide samples (50–100 pmol) in 1–2 μL of 5% aqueous acetic acid were added to 1–2 μL of a mixture of DTT/DTE (5/1) on a 1.5 × 6 mm stainless steel sample stage. Repetitive scans over the mass range m/z 30–3000 (cycle time = 45 s) were collected by using a JEOL DA5000 data system.

FD mass spectra were obtained with activated carbon emitter. Samples were dissolved in a few microliters of methanol and applied with a syringe. Instrument resolution was set at 1000 and data were collected over the mass range m/z 30–1000. Mass values reported are for the monoisotopic mass of the protonated molecular ion rounded off to the nearest integral mass.

RESULTS

Enzyme Purification. DT-diaphorase was isolated from livers of 3-methylcholanthrene-induced rats. By use of azodicoumarol affinity chromatography, a single homogeneous enzyme was obtained in a yield of 70% from starting materials. SDS gel electrophoresis of the enzyme preparation exhibited a single band at a molecular mass of 32 000 Da (data not shown). The purified enzyme had a similar absorption spectrum to that reported by Höjeberg et al. (1981).

Peptide Maps. Tryptic digests of S-carboxymethylated DT-diaphorase were loaded onto an Ultrasphere C8 reverse-phase column (Figure 1 in supplementary material). Radio-

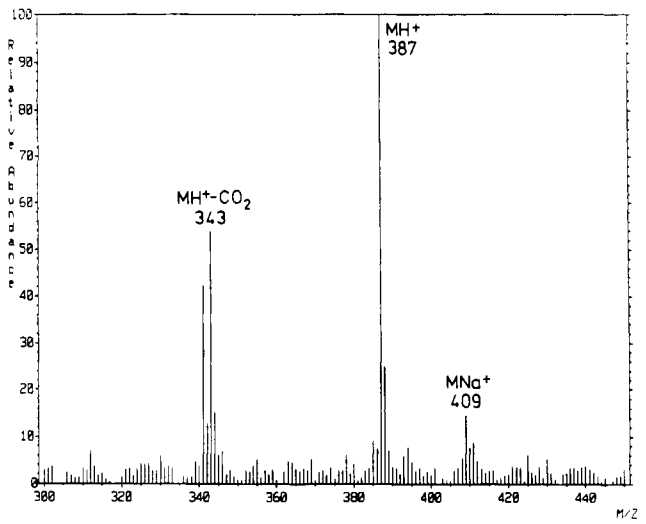

FIGURE 1: Field desorption mass spectrum of peptide T-1. Both the protonated molecular ion (m/z 387) and the molecular ion plus sodium (m/z 409) were observed. The prominent ion at m/z 343 is consistent with loss of CO_2 as the sample was heated on the emitter.

activity was mainly detected in peak T-14, corresponding to the only cysteine-containing peptide in the protein. A tryptic core was dissolved in hexafluoroacetone trihydrate (HFA) and chromatographed on a μBondapak phenyl column (Figure 2 in supplementary material). Three major peaks were recovered. Amino acid compositions of all of the tryptic peptides are shown in Table I of the supplementary material. The peptide maps of chymotryptic and *S. aureus* protease digests were obtained by similar methods (Figure 3 in supplementary material). However, some insoluble core materials were observed for the *S. aureus* protease digest. Cyanogen bromide fragments provided peptides soluble in 70% formic acid and were separated on a Vydac C4 column (Figure 4 in supplementary material). Amino acid compositions of cyanogen bromide peptides are shown in Table II of the supplementary material.

Sequence Determination. Protein sequence analysis by automated and manual Edman degradation demonstrated that the amino terminus of DT-diaphorase was blocked. Peptide T-1 was shown to be an N-blocked peptide consisting of three amino acids, Ala, Val, and Arg (Table I in supplementary material). FD/MS analysis of this peptide revealed a protonated molecular ion (MH^+) of mass 387, suggesting that the N-blocking group was an acetyl group (Figure 1) since the calculated value of the protonated molecular ion for the indicated sequence was 345. COOH-terminal sequence analysis of peptide T-1 by CPase Y digestion showed the sequence -Val-Arg. Thus, the sequence of this peptide was determined to be CH_3CO-Ala-Val-Arg. This result was confirmed by amino acid composition and FAB/MS analysis of CNBr peptide CB-1. An MH^+ = 2383 was observed for this peptide in the homoserine lactone form. The difference (42) between the observed mass and that for the lactone form was once again consistent with an NH_2-terminal acetyl group. A summary of the sequence analyses of NH_2-terminal peptides is given in Table III of the supplementary material. The tryptic core contained mainly three large peptides, including the highly hydrophobic and aromatic amino acid rich peptide T-10. Alignment of the tryptic fragments required further proteolytic fragmentation and chemical cleavage (Table III in the supplementary material). Although *S. aureus* protease digestion provided many of the overlapping peptides, sequence analysis of several cyanogen bromide peptides and some of the chymotryptic peptides was necessary to complete the sequence

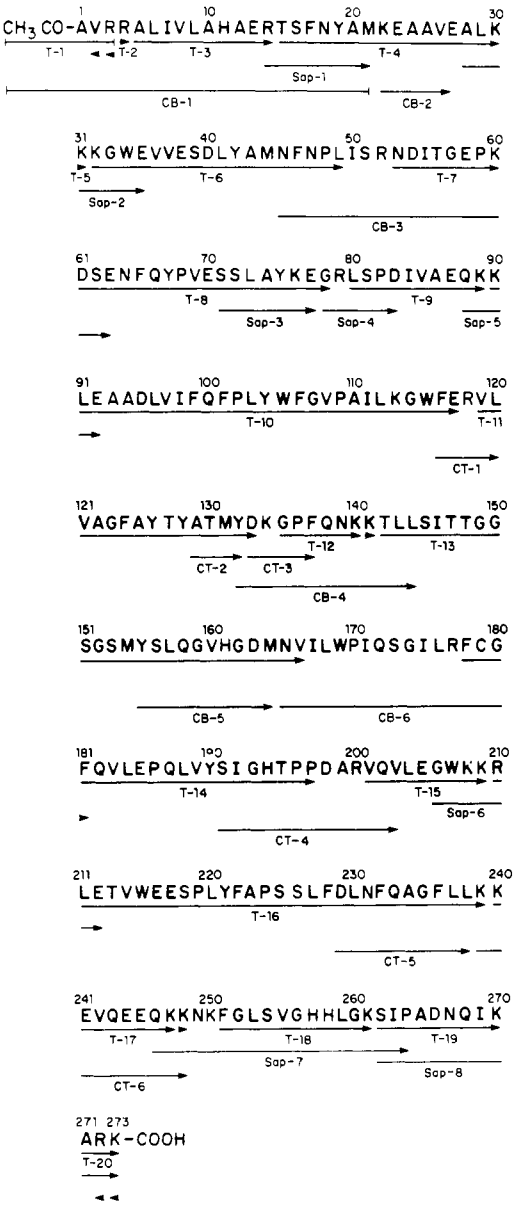

FIGURE 2: Total amino acid sequence of rat liver DT-diaphorase. Arrows (→) under the sequence denote the residues determined by Edman degradations. Reverse arrows (←) show the residues analyzed by CPase digestion. TNBS-labeled lysine is located at residue 76. Essential tyrosine probably interacting with the substrate is located at residue 128 in the highly hydrophobic region.

for DT-diaphorase. We have isolated NH_2-terminal cyanogen bromide peptide CB-1, which could not be detected previously by Bayney et al. (1987). The complete amino acid sequence of DT-diaphorase is shown in Figure 2.

Inhibition Studies with NBSF. DT-diaphorase was strongly inhibited by NBSF (0–30 μM), exhibiting a time-dependent and concentration-dependent loss of the enzyme activity. The inhibition exhibited pseudo-first-order kinetics as shown in Figure 3. The pseudo-first-order rate constant (k_1) of inactivation was calculated from the plot of log (enzyme activity) vs time. The slope showed the rate constant k_1. The second-order rate constant (k_2) was obtained from the slope of a double-reciprocal plot of $1/k_{obsd}$ vs $1/[NBSF]$ (k_2 = 0.32 min^{-1} M^{-1}). The reaction order (n = 1.1) was obtained from the slope of log (k_1) vs log [NBSF] from the following formula: $\log k_{obsd} = \log k_2 + n \log [NBSF]$ (Palczewski et al., 1985).

Protection with Effectors on NBSF Inhibition. The NBSF inhibition was performed in the presence of several substrate analogues such as 1-naphthol or 1-naphthylamine. As shown

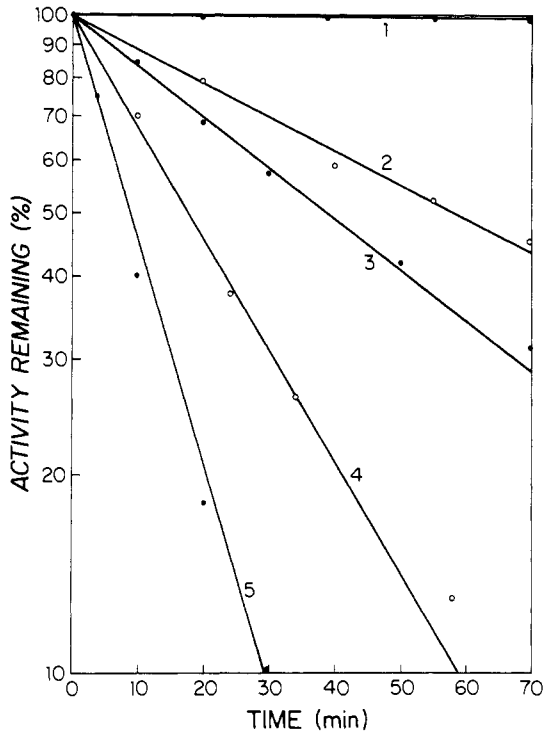

FIGURE 3: Time-dependent and concentration-dependent inhibition of DT-diaphorase with *p*-nitrobenzenesulfonyl fluoride (NBSF). The enzyme (0.65 μM) was incubated with various concentrations of NBSF at room temperature: (1) 0, (2) 3, (3) 6, (4) 13, and (5) 30 μM, respectively.

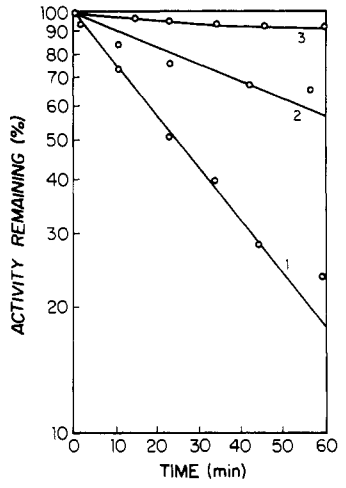

FIGURE 4: Protection effect of naphthalene derivatives on NBSF inhibition. The DT-diaphorase (0.8 μM) was incubated with NBSF (6 μM) in the presence or absence of effectors: (1) no effector; (2) 1-naphthylamine (0.4 mM); (3) 1-naphthol (0.4 mM).

in Figure 4, both 1-naphthol and 1-naphthylamine exhibited a strong protection effect against NBSF inhibition.

Inhibition with TNBS. Inhibition of DT-diaphorase with TNBS resulted in a time-dependent and concentration-dependent loss of the enzyme activity. The inhibition followed pseudo-first-order kinetics (Figure 5). The first-order rate constant (k_1) of inactivation was calculated from the slope of a plot of log (enzyme activity) (%) vs time. The second-order rate constant (k_2) was calculated from the slope of a double-reciprocal plot of $1/k_{obsd}$ vs $1/[TNBS]$. A value of k_2 = 0.0142 min^{-1} M^{-1} was obtained. The reaction order (n = 0.62) was obtained from the slope according to the above formula (Palczewski et al., 1985).

Protection Effects of NADH, NAD$^+$, and Effectors on TNBS Inhibition. The enzyme was incubated with TNBS in

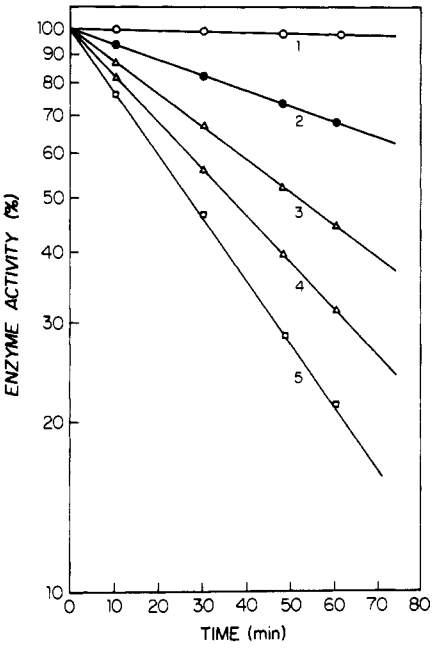

FIGURE 5: Time-dependent and concentration-dependent inhibition with 2,4,6-trinitrobenzenesulfonate. The enzyme (0.7 μM) was incubated with various concentrations of TNBS solutions at room temperature: (1) 0, (2) 3, (3) 13, (4) 26, and (5) 50 μM, respectively.

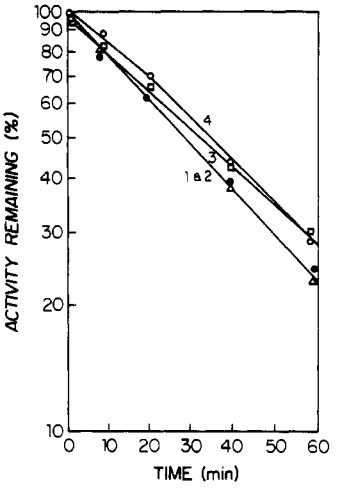

FIGURE 6: Effect of naphthalene derivatives on TNBS inhibition. The enzyme (0.7 μM) was incubated with naphthalene derivatives (0.4 mM) prior to TNBS labeling: (1) control (no effector); (2) 1-naphthol; (3) 1-naphthylamine; (4) 1-naphthlenesulfonic acid.

the absence or presence of NAD$^+$ or NADH. NAD$^+$ and NADH did not prevent the inhibition of enzyme activity by TNBS. An initial decrease in the enzyme activity was observed when the enzyme was preincubated with NADH prior to TNBS inhibition (data not shown), probably because of the conformational change of the enzyme or reaction between TNBS and NADH. Naphthalene derivatives such as 1-naphthol or 1-naphthylamine also did not prevent the TNBS inhibition as shown in Figure 6. These results suggest that the essential lysine residue may not be present in the substrate- or NADH-binding regions, but is probably in the electron-transferring site.

Stoichiometry of TNBS Labeling. The stoichiometry of lysine residues reactive to TNBS vs inhibition of enzyme activity was studied. Since 2.5–2.7 mol of modified lysine residues/mol of enzyme was obtained by two separate experiments, one subunit contained 1.2–1.3 of reactive lysine residues. The stoichiometry of TNBS labeling in the presence of me-

Table I: Sequence Analyses of the NBSF-Labeled Peptides[a]

	NBS-1 (250 pmol)			NBS-2 (210 pmol)		
cycle	residue	PTH	pmol	residue	PTH	pmol
1	127	Thr	80	125	Ala	160
2	128	Tyr	*b*	126	Tyr	103
3	129	Ala	200	127	Thr	30
4	130	Thr	60	128	Tyr	*b*
5	131	Met	150	129	Ala	80
6	132	Tyr	140	130	Thr	22
7	133	Asp	130	131	Met	64
8	134	Lys	+	132	Tyr	57
9				133	Asp	62
10				134	Lys	+

[a] Yields of the modified peptides were 625 (NBS-1) and 525 pmol (NBS-2), respectively. [b] PTH-tyrosine was not detected due to the NBSF modification.

nadione or dicoumarol was determined to be 0.7 or 0.5 mol/subunit.

Isolation of NBSF-Labeled Peptide and the Sequence Determination. DT-diaphorase (10 nmol) was incubated with 10 μM NBSF for 1 h at 25 °C. After removal of excess reagent by Sephadex G-25 chromatography, the sample was trypsinized, followed by HPLC analysis using a Vydac C4 column, which permits a recovery of hydrophobic or large peptides (Figure 7). The NBSF-labeled peptides were tentatively identified as those whose UV absorption at 254 nm exceeded that at 280 nm. After microsequence analysis, two peaks were designated at NBS-peptides, NBS-1 and NBS-2 (Table I). Other peaks did not sequence and were assumed to be byproducts of the NBSF derivatization. Peptide NBS-1 was shown to be T-Y-A-T-M-Y-D-K (residues 127–134) by microsequence analysis. A low recovery of the PTH derivative was obtained for cycle 2 (Table II). Because the yield of tyrosine PTH for the sixth cycle was consistent with the remainder of the run, the cycle 2 residue was assumed to be labeled with NBSF. Peptide NBS-2 confirms this assignment; the sequence of this peptide was determined to be A-Y-T-Y-A-T-M-Y-D-K (residues 125–134). Both peptides were likely derived from combined tryptic and chymotryptic action, probably because the trypsin used is slightly contaminated with chymotrypsin. The tyrosine expected at the fourth cycle in this peptide was not detected by microsequence analysis. Once again this is consistent with the modification by NBSF. These results indicate that tyrosine-128 was the only site labeled with NBSF and that this tyrosine is essential for enzyme activity.

Isolation and Sequence Analysis of TNBS-Labeled Peptides. DT-diaphorase was incubated with TNBS in the presence or absence of dicoumarol. The protein samples were trypsinized, and the resulting peptides were separated on a Vydac C4 column. The digested materials were totally soluble in 0.2 M ammonium bicarbonate buffer, pH 8.0. The results are shown in Figure 8. One major TNBS-labeled peptide TNBS-1 and other minor peptides TNBS-2 and TNBS-3 were indicated by high absorption at 350 nm. Although other peaks absorbing at 350 nm were also observed, these peaks were probably derived from TNBS or the breakdown products because no peptides were detected after amino acid analysis and sequence analysis of these peaks. Sequence analysis gave the sequence Lys-Glu-Gly-Arg (residues 76–79) for peptide TNBS-1. Lysine-76 might be modified with TNBS because of the low yield for the lysine at cycle 1. Amino acid composition and sequence analysis data of this peptide are shown in Tables II and III. The other minor peptides TNBS-2 (Tables II and III) and TNBS-3 (data not shown) span residues 61–79. FAB/MS analysis of TNBS-2 revealed the covalent linkage of the TNP moiety to the peptide.

Table II: Amino Acid Compositions of TNBS-Labeled Peptides[a]

amino acid	TNBS-1	TNBS-2
Asp		2.1 (2)
Thr		
Ser		2.4 (3)
Glu	1.0 (1)	3.6 (4)
Pro		1.0 (1)
Gly	1.1 (1)	1.2 (1)
Ala		1.1 (1)
Val		1.0 (1)
Met		
Ile		
Leu		1.1 (1)
Tyr		1.6 (2)
Phe		0.9 (1)
Lys[b]	0.2 (1)	0.3 (1)
His		
Arg	0.9 (1)	0.9 (1)
total	4	19
yield (pmol)	610	240

[a] Number in parentheses denotes residues determined from sequence analysis. [b] ε-TNP-Lys is resistant to HCl hydrolysis.

Table III: Sequence Analysis of TNBS-Labeled Peptides

	TNBS-1 (410 pmol)			TNBS-2 (150 pmol)		
cycle	residue	PTH	pmol	residue	PTH	pmol
1	76	Lys	*a*	61	Asp	64
2	77	Glu	210	62	Ser	14
3	78	Gly	132	63	Glu	70
4	79	Arg	43	64	Asn	61
5				65	Phe	48
6				66	Gln	43
7				67	Tyr	22
8				68	Pro	9
9				69	Val	24
10				70	Glu	19
11				71	Ser	3
12				72	Ser	5
13				73	Leu	11
14				74	Ala	10
15				75	Tyr	6
16				76	Lys	*a*
17				77	Glu	5
18				78	Gly	5
19				79	Arg	+

[a] PTH-lysine was not detected due to trinitrophenylation of the ε-NH_2 group.

Discussion

The NH_2-terminal sequence analysis of DT-diaphorase was unsuccessful by Edman degradation since it was blocked with an unknown group (Bayney et al., 1987). From this study, we have isolated the N-blocking peptide and determined the *N*-acetyl moiety by mass spectrometric analysis of the tryptic and cyanogen bromide fragments. A comparison of our protein sequence analysis with that from the cDNA sequence analysis (Robertson et al., 1986) suggests that the initial methionine was cleaved during biosynthesis and the resulting NH_2-terminal alanine might be acetylated in a similar manner observed for NADPH–cytochrome P-450 reductase (Haniu et al., 1986). However, DT-diaphorase does not contain a hydrophobic signal peptide as is found for other membrane proteins, suggesting a cytosolic location of this enzyme. There is some sequence similarity between DT-diaphorase and ferredoxin–$NADP^+$ reductase: Triple lysines at residues 30–32 were surrounded by double glutamic acids. A similar sequence was also found in the NH_2-terminal region of NADPH–cytochrome P-450 reductase.

A comparison of the sequences deduced from cDNAs by two groups (Robertson et al., 1986; Bayney et al., 1987) reveals

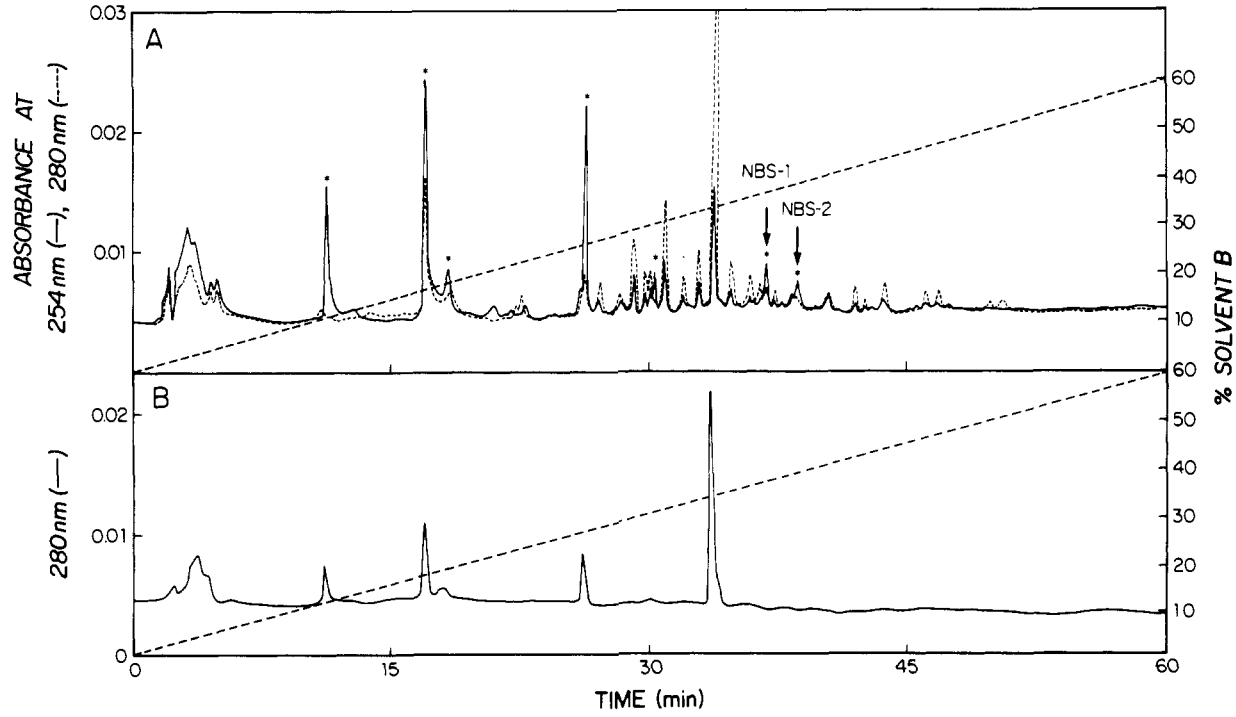

FIGURE 7: Isolation of NBSF-labeled peptides. (A) DT-diaphorase was incubated with 100-fold molar excess of NBSF at room temperature. After measurement of enzyme activity, the reaction mixture was desalted by Sephadex G-25 column chromatography. The modified protein was trypsinized, followed by HPLC separation on a Vydac C4 column. The NBSF-peptides were detected as a 2–3-fold higher absorption peak at 254 nm than at 280 nm. Asterisks except for NBS-1 and -2 show the peaks derived from the NBSF reagent. (B) NBSF background.

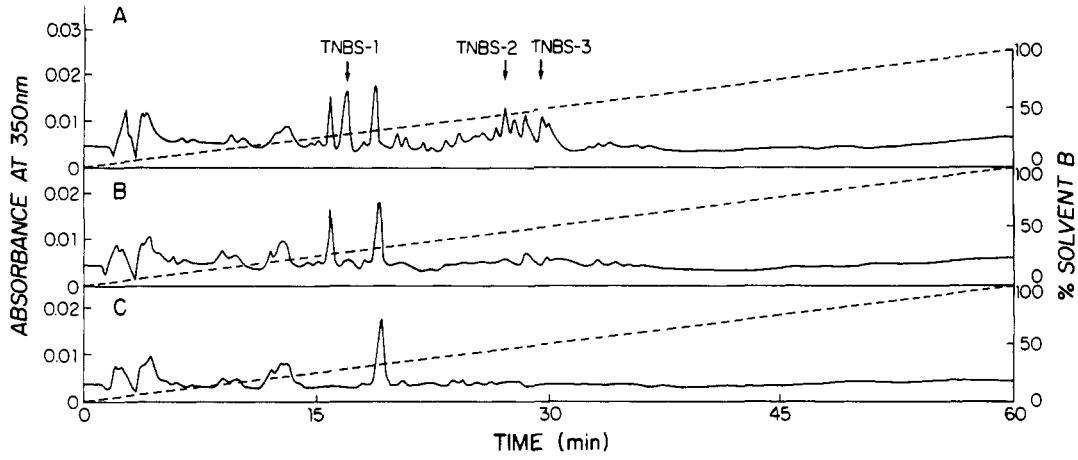

FIGURE 8: Isolation of TNBS-modified peptides by HPLC. The enzyme was modified with a 100-fold molar excess of TNBS, digested with TPCK–trypsin, and analyzed by HPLC using a Vydac C4 column (4.6 × 250 mm). (A) Modified enzyme, (B) enzyme modified in the presence of dicoumarol, and (C) TNBS background.

that one is 20 amino acid residues shorter than the other. Our sequence data completely match the sequence reported by Robertson et al. (1986) except for the methionine at the NH_2 terminus. Although Bayney et al. (1987) attempted protein sequence analysis, they likely missed the N-terminal peptide due to the blocked NH_2 terminus.

The subunit of DT-diaphorase contains a single cysteine residue. However, it is not clear whether it exists as a free sulfhydryl residue or a disulfide bond between two subunits. According to Huang et al. (1979) and Höjeberg et al. (1981), the disulfide linkage may exist in the native state because the enzyme behaves as a monomer under the reducing condition of SDS–PAGE. However, we have isolated a radiolabeled (carboxymethyl)cysteine from the native enzyme that was S-alkylated with [^{14}C]iodoacetate under nonreducing conditions, suggesting that the disulfide linkage between two subunits is unlikely. When we modified the enzyme with 2 mM *N*-ethylmaleimide (for 1 h), the enzyme retained full activity. Further treatment with TNBS gave complete inactivation. These results support the idea that if the SH group exists, it is not essential for the enzyme activity.

Naphthalene derivatives clearly protected the enzyme from NBSF inhibition. The substrate binding region can be expected to lie in highly hydrophobic or aromatic amino acid rich regions. Examination of these regions suggests that one region (residues 96–132) is a likely candidate for the substrate binding site. This proposal is consistent with our results. However, naphthalene derivatives were not able to prevent TNBS inhibition of the enzyme. Therefore, the functional role of the essential lysine residue (Lys-76) is still unclear.

There could be several functional domains in this enzyme: FAD binding, NAD(P)H binding, and substrate or dicoumarol binding domains. Sequence homology analysis does not show any similarity with other flavoproteins (Robertson et al., 1986). However, it can be expected that the nucleotide binding site is located in the hydroxyamino acid or glycine-rich regions

(Porter & Kasper 1986; Haniu et al., 1986). The region from residues 144 to 158 (three threonines, four serines, and three glycines) is a candidate for the NAD(P)H- or FAD-binding region.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of James A. Sligar for amino acid analysis and PTH analysis and Kassu Legesse for mass spectrometry.

SUPPLEMENTARY MATERIAL AVAILABLE

Tables of amino acid compositions and sequence analyses and figures of chromatographic analysis of the peptide fragments (11 pages). Ordering information is given on any current masthead page.

Registry No. NBSF, 349-96-2; TNBS, 2508-19-2; DT-diaphorase, 9032-20-6; DT-diaphorase (rat liver), 115756-48-4; tyrosine, 60-18-4; lysine, 56-87-1.

REFERENCES

Bayney, R. M., Rodkey, J. A., Bennett, C. D., Lu, A. Y. H., & Pickett, C. B. (1987) *J. Biol. Chem. 262*, 572–575.
Del Valle, U., & Shively, J. E. (1979) *Anal. Biochem. 96*, 77–83.
Ernster, L., Ljunggren, M., & Danielson, L. (1960) *Biochem. Biophys. Res. Commun., 2*, 88–92.
Ernster, L., Danielson, L., & Ljunggren, M. (1962) *Biochim. Biophys. Acta 58*, 171–188.
Ferrante, R. J., Kowall, N. W., Beal, M. F., Richardson, E. P., Jr., Bird, E. D., & Martin, J. B. (1985) *Science (Washington, D.C.) 230*, 561–563.
Fields, R. (1971) *Biochem. J. 124*, 581–590.
Haniu, M., Iyanagi, T., Miller, P., Lee, T. D., & Shively, J. E. (1986) *Biochemistry 25*, 7906–7911.
Hawke, D. H., Yuan, P.-M., & Shively, J. E. (1982) *Anal. Biochem. 120*, 302–311.
Hawke, D. H., Harris, D. C., & Shively, J. E. (1985) *Anal. Biochem. 147*, 315–330.
Höjeberg, B., Blomberg, K., Stenberg, S., & Lind, C. (1981) *Arch. Biochem. Biophys. 207*, 205–216.
Hollander, P. M., & Ernster, L. (1975) *Arch. Biochem. Biophys. 169*, 560–567.
Hosoda, S., Nakamura, W., & Hayashi, K. (1974) *J. Biol. Chem. 249*, 6416–6423.
Huang, M.-T., Miwa, G. T., & Lu, A. Y. H. (1979) *J. Biol. Chem. 254*, 3930–3934.
Iyanagi, T. (1987) *Chem. Scr. 27A*, 31–36.
Iyanagi, T., & Yamazaki, I. (1970) *Biochim. Biophys. Acta 216*, 282–294.
Koh, J.-Y., Peters, S., & Choi, D. W. (1986) *Science (Washington, D.C.) 234*, 73–76.
Liao, T.-H., Ting, R. S., & Yeung, J. E. (1982) *J. Biol. Chem. 257*, 5637–5644.
Lind, C., Hochstein, P., & Ernster, L. (1982) *Arch. Biochem. Biophys. 216*, 178–185.
Miller, M. G., Rodgers, A., & Cohen, G. M. (1986) *Biochem. Pharmacol. 35*, 1177–1184.
Palczewski, K., Hargrave, P. A., Folta, E. J., & Kochman, M. (1985) *Eur. J. Biochem. 146*, 309–314.
Porter, T. D., & Kasper, C. B. (1986) *Biochemistry 25*, 1682–1687.
Prochaska, H., & Talalay, P. (1986) *J. Biol. Chem. 261*, 1372–1378.
Robertson, J. A., Chen, H.-C., & Nebert, D. W. (1986) *J. Biol. Chem. 261*, 15794–15799.
Shively, J. E., Yuan, P.-M., & Hawke, D. H. (1982) *Anal. Biochem. 120*, 312–322.
Talalay, P., & Benson, A. M. (1980) *Adv. Enzyme Regul. 20*, 287–300.
Thor, H., Smith, M. T., Hartzell, P., Bellomo, G., Jewell, S. A., & Orrenius, S. (1982) *J. Biol. Chem. 257*, 12419–12425.
Wallin, R., Gerhardt, O., & Prydz, H. (1978) *Biochem. J. 169*, 95–101.
Williams, J. B., Lu, A. Y. H., Cameron, R. G., & Pickett, C. B. (1986) *J. Biol. Chem. 261*, 5524–5528.

Structure–Activity Relationships of Recombinant Human Interleukin 2

M. P. Weir,* M. A. Chaplin, D. M. Wallace, C. W. Dykes, and A. N. Hobden
Genetics Unit, Glaxo Group Research Ltd., Greenford Road, Greenford, Middlesex UB6 0HE, U.K.
Received March 14, 1988

ABSTRACT: Structure–activity relationships of recombinant human interleukin 2 were investigated by preparation, purification, and characterization of 21 missense mutants. A key role for residue Phe42 in the high-affinity interaction with receptor was indicated by (a) the reduction of 5–10-fold in binding affinity and bioactivity upon mutation of this residue to Ala and (b) the lack of evidence for conformational perturbation in Phe42 → Ala in comparison with the wild-type protein as investigated by intrinsic fluorescence, second-derivative UV spectroscopy, electrophoresis, and reversed-phase HPLC, suggesting that the drop in binding is a direct effect of removal of the aromatic ring. In contrast, the conservative mutations Phe42 → Tyr and Phe42 → Trp did not cause significant reductions in bioactivity. UV and fluorescence spectra indicated approximately 60% overall exposure to solvent of tyrosines in the wild-type molecule, the tryptophan (residue 121) being buried; fluorescence data also showed that Trp42 in Phe42 → Trp is likely to be within 1 nm of Trp121 and about 50% exposed to solvent. Phe44 → Ala, Cys105 → Ala, and Trp121 → Tyr also exhibited reduced bioactivity, but these mutants are conformationally perturbed relative to wild type. None of the remaining mutants had detectably reduced bioactivity, even though several showed signs of altered conformation. Four mutants were recovered in very low yield, probably because of defective refolding.

Interleukin 2 (IL-2)[1] is a 133-residue protein secreted by T-lymphocytes that promotes the proliferation of activated T-helper cells (Robb, 1985) and modulates growth and differentiation of other lymphocyte subsets, for example, activated

0006-2960/88/0427-6883$01.50/0