Some Molecular Properties of NAD(P)H Dehydrogenase from Rat Liver

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NAD(P)H dehydrogenase (EC 1.6.99.2) purified from rat liver cytosol revealed three discrete bands, of mol.wts. about 27000, 18000 and 9000, when subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Elution of the bands from the gel and individual re-electrophoresis on separate gels showed that the 27000-mol.wt. band yielded three bands similar to those obtained with the intact enzyme, whereas the 18000-mol.wt. band retained its characteristic mobility. Amino acid analysis of native enzyme and protein extracted from each of the three bands from sodium dodecyl sulphate/polyacrylamide gels suggests that the native enzyme is composed of two subunits and that each subunit consists of two dissimilar non-covalently bound polypeptides, so that altogether the enzyme is composed of four polypeptides, two of mol.wt. 18000 and two of mol.wt. 9000. NAD(P)H dehydrogenase was active over a wide pH range with no sharp optimum. The same K_m value for NADH but different values for V_{max} were obtained for the enzyme purified from Sprague–Dawley and Wistar rats. In immunodiffusion, however, the enzymes from the two rat strains showed a reaction of complete identity. NAD(P)H dehydrogenase was effectively inhibited by thiol-blocking reagents, indicating that the activity is dependent on free thiol group(s). By amino acid analysis six cysteine residues were found per mol of enzyme. Guanidino-group- and amino-group-selective reagents had only moderate inactivating effects on the enzyme activity.

NAD(P)H dehydrogenase (EC 1.6.99.2) is an enzyme found both in the liver cytosol and mitochondrial membranes catalysing the oxidation of NADH and NADPH by various electron acceptors (Ernster & Navazio, 1958; Ernster, 1958, 1967). We have recently established a simple purification scheme for this enzyme from rat liver by affinity chromatography and studied its role in the vitamin K-dependent carboxylation reaction (Wallin *et al.*, 1978; Wallin, 1979). The enzyme acts by producing reduced vitamin K which is active in this reaction (Sadowski *et al.*, 1976; Friedman & Shia, 1976; Girardot *et al.*, 1976).

The kinetics of the enzyme have been studied by several groups (Hosoda *et al.*, 1974; Rase *et al.*, 1976; Ernster *et al.*, 1962, 1972), but very little is known about its molecular properties.

Rase *et al.* (1976) purified the enzyme from Sprague–Dawley rats. It was found to have a mol.wt. of 55000 by gel filtration, but dissociated into two subunits of similar mol.wt. (27000) when subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. We (Wallin *et al.*, 1978) obtained similar results for the enzyme purified from Wistar rats and

concluded that the two subunits were not identical on the basis of results from *N*-terminal analysis. The present paper describes some further molecular properties of NAD(P)H dehydrogenase purified from livers of Wistar rats.

Lind *et al.* (1973) reported a significantly higher activity of NAD(P)H dehydrogenase in Sprague–Dawley rats than in Wistar rats. Some comparisons have therefore been made between the enzymes purified from the two sources.

Materials and Methods

Animals

Male Wistar–Møll rats and male Sprague–Dawley rats (Møllgaard, Ejby, Denmark) were fed on a vitamin K-deficient diet while they were kept in coprophagy-preventing cages as described by Wallin *et al.* (1978).

Chemicals

Chemicals were obtained as follows: DEAE-Sephadex A-50 was from Pharmacia, Uppsala, Sweden. Agarose (Indubiose A 37) was from L'Industrie Biologique Francaise S.S., France. Polyacrylamide was from Eastman Organic Chemicals, Rochester, NY, U.S.A. *NN'*-Methylene-bisacrylamide was from Merck, Darmstadt, Germany.

Abbreviations used: SDS, sodium dodecyl sulphate; when not specified in the text NAD(P)H dehydrogenase refers to the enzyme purified from Wistar rats.

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NNN'-Tetramethylethylenediamine, 2,6-dichlorophenol-indophenol (sodium salt), Tween 20 and 1,2-cyclohexanedione were from Koch-Light, Colnbrook, Bucks., U.K. Cyclohexanedione was recrystallized from diethyl ether. SDS was from BDH, Poole, Dorset, U.K. β -NADH, 2,4,6-trinitrobenzenesulphonic acid, pyridoxal 5'-phosphate, bovine serum albumin, ovalbumin, myoglobin and insulin were from Sigma Chemical Co., St. Louis, MO, U.S.A. 5,5'-Dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co., Milwaukee, WI, U.S.A., and 2,3-butanedione was from Fluka, Buchs, Switzerland. Butanedione was redistilled before use. Methanesulphonic acid (4M), containing 0.2% 3-(2-aminoethyl)indole, was from Pierce Chemical Co., Rockford, IL, U.S.A. Immunoglobulin G was from Kabi, Stockholm, Sweden. Basic Fuchsin was from Matheson, Coleman and Bell, Norwood, OH, U.S.A. Sodium warfarin [4-hydroxy-3-(3-oxo-1-phenylbutyl)coumarin] was from Nyegaard, Oslo, Norway. All other chemicals were reagent-grade or better.

Purification of NAD(P)H dehydrogenase

NAD(P)H dehydrogenase was purified from rat liver cytosol by affinity chromatography on Sepharose-menadione as previously described (Wallin *et al.*, 1978). Enzyme released from the affinity column in 50mM-Tris/HCl (pH 7.2) containing 50% dioxan was desalted on Sephadex G-25 in 50mM-Tris/HCl (pH 8.6). The void-volume fractions containing the enzyme were pooled and applied to a DEAE-Sephadex A-50 ion exchanger equilibrated in the same buffer. Elution of the enzyme from the column was performed with a gradient of NaCl (0–500mM) in the equilibration buffer by using an Ultrograd 11300 gradient-mixer (LKB, Bromma, Sweden) (see Fig. 1).

Electrophoretic methods

Analytical polyacrylamide-gel electrophoresis was performed at pH 9.4 as described by Davis (1964) and at pH 4.3 as described by Maurer (1971). SDS/polyacrylamide-gel electrophoresis was carried out by the method of Laemmli (1970) in 7% gels. Before electrophoresis the sample was kept at 100°C for 3 min in the electrode buffer containing 1% SDS. Gels were stained for protein with Coomassie Brilliant Blue R-250 and for carbohydrates (periodate/Schiff staining) as described by Bjørklid *et al.* (1973) and Segrest & Jackson (1972) respectively. For molecular-weight estimation, ovalbumin, bovine serum albumin and the heavy and light chains of immunoglobulin G were used as standards. Protein bands were cut from unstained gels according to their R_F values and eluted in distilled water.

Amino acid analysis

Quantitative amino acid analysis was carried out as described by Spackman *et al.* (1958) using a JEOL JLC-6AH amino acid analyser. Protein (samples of 250 μ g) was hydrolysed *in vacuo* in constant-boiling HCl at 105°C for 24, 48 and 72 h. The values for threonine and serine were obtained by extrapolation to zero hydrolysis time. Half-cystine was determined as cysteic acid after performate oxidation (Hirs, 1956). For tryptophan determination protein was hydrolysed *in vacuo* for 22 h in 4M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole as described by Simpson *et al.* (1976).

Antiserum

NAD(P)H dehydrogenase purified from Wistar rats was used to raise antibodies in a rabbit. Enzyme (0.1 mg) mixed with Freund's adjuvant was given at multiple subcutaneous sites twice with an interval of 1 month; 1 week after the last injection 30 ml of blood was drawn from the rabbit. Portions of antiserum were frozen and stored at –20°C.

Immunological methods

Immunodiffusion and immunoelectrophoresis in 1% agarose were carried out as described by Ouchterlony (1948) and Scheidegger (1955) respectively.

Measurements of enzyme activity

NAD(P)H dehydrogenase was assayed as described by Dallner (1963) with 2,6-dichlorophenol-indophenol as hydrogen acceptor at room temperature. The reaction was followed at 600 nm and the enzyme activity calculated by using a molar absorption coefficient for 2,6-dichlorophenol-indophenol of $21 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$. For Lineweaver-Burk plots, the concentration of NADH was varied while the concentration of 2,6-dichlorophenol-indophenol was kept at 40 μ M.

Modification of amino acid side chains

Enzyme activity was measured 1 min after the addition of the thiol-blocking reagents HgCl_2 and 5,5'-dithiobis-(2-nitrobenzoic acid). The effect of all other reagents on enzyme activity was followed continuously over a period of 20 min at 22°C.

For amino groups pyridoxal 5'-phosphate and 2,4,6-trinitrobenzenesulphonic acid were used as selective reagents (Ernest & Kim, 1974; Means & Feeney, 1971), and for guanidino groups 2,3-butanedione and 1,2-cyclohexanedione were used (Patthy & Smith, 1975a,b; Sairam, 1976).

Other methods

Protein was measured by the filter method of Schaffner & Weissmann (1973), with bovine serum albumin as standard. Alkylation by iodoacetate was carried out as described by Ramachandran & Colman (1978). FAD was detected by its fluorescence at 535 nm by using an excitation wavelength of 450 nm (Faeder & Siegel, 1973) in a Perkin-Elmer fluorescence spectrophotometer MPF-3. Alternatively, the flavin content was determined by the absorbance at 450 nm [absorption coefficient for FAD, $113 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Whitby, 1953)]. All other spectrophotometric measurements were performed in a Beckman model 25 spectrophotometer with an automatic recorder.

Results

The active fractions (3 ml) from the DEAE-Sephadex chromatography (Fig. 1) were pooled and dialysed against $2.5 \text{ mM-NH}_4\text{HCO}_3$. The enzyme was subsequently concentrated by freeze-drying and dissolved in a small volume of $2.5 \text{ mM-NH}_4\text{HCO}_3$. Such preparations gave one band in analytical disc gels at pH 9.4 (Fig. 6).

SDS/polyacrylamide-gel electrophoresis of NAD(P)H dehydrogenase

When NAD(P)H dehydrogenase was submitted to SDS/polyacrylamide-gel electrophoresis two

Fig. 1. Ion-exchange chromatography of NAD(P)H dehydrogenase isolated by affinity chromatography on Sepharose-menadione

A column ($1.8 \text{ cm} \times 12 \text{ cm}$) of DEAE-Sephadex A-50 was equilibrated in 50 mM-Tris/HCl , pH 8.6. Desalted enzyme from a Sepharose-menadione column was loaded on to the column, which was eluted by a gradient of NaCl ($0\text{--}500 \text{ mM}$) in the equilibration buffer by using an Ultrograd 11300 gradient mixer. The flow rate was 34 ml/h . Absorbance of eluate (—) was monitored with a Uvicord III 2098 u.v.-absorptiometer. ●, NAD(P)H dehydrogenase activity. ----, NaCl gradient.

major protein bands and one weaker band moving close to the marker were seen (Fig. 2a). Reduction by mercaptoethanol before electrophoresis did not change the electrophoretic picture. The molecular weights corresponding to the three bands were estimated from the mobilities of standard proteins to be about 27 000 for band I, 18 000 for band II and in the range 8000–9000 for band III.

Bands I and II from five replicate gels were cut out, eluted in distilled water and dialysed against the electrode buffer containing 1% SDS. After concentration, extracts from bands I and II were re-electrophoresed separately. Band I yielded three protein bands (Fig. 2b) similar to those obtained with the intact enzyme (Fig. 2a). Band II, however, retained its characteristic mobility and only one band was seen (Fig. 2b). Identical results were obtained with NAD(P)H dehydrogenase purified from livers of Sprague-Dawley rats (not shown).

Attempts to convert band I (mol.wt. 27 000) completely into bands II (mol.wt. 18 000) and III (mol.wt. 8000–9000) by prolonged boiling before electrophoresis were unsuccessful. The presence of $6 \text{ M-guanidine hydrochloride}$, 1% SDS and 1% dithiothreitol during the boiling had no detectable effect, nor did alkylation of the enzyme change the pattern (Fig. 2a).

Staining for glycoprotein by the periodate/Schiff procedure was completely negative. No colour

Fig. 2. SDS/polyacrylamide gel electrophoresis of NAD(P)H dehydrogenase in 7% gels

(a) Purified NAD(P)H dehydrogenase ($15 \mu\text{g}$); (b) protein bands I and II from five polyacrylamide gels were cut out, eluted and re-electrophoresed individually. A, band I; B, band II. M, marker.

developed, even when 20 μ g of intact enzyme had been submitted to electrophoresis. Prothrombin fragment I (Wallin & Prydz, 1977) was used as a control and for this protein fragment the staining for carbohydrate was found to be about as sensitive as staining for protein with Coomassie Brilliant Blue R-250.

By its fluorescence, FAD was found to be present in extracts from SDS/polyacrylamide gels of bands I and II, but not in band III.

Gel filtration in guanidine hydrochloride

The elution pattern of NAD(P)H dehydrogenase from a column of Sephadex G-75 in 50mM-potassium phosphate (pH 7.5) containing 6M-guanidine hydrochloride is shown in Fig. 3. The enzyme, which before chromatography had been incubated for 15 min at 37°C, exhibited three peaks. By comparing the elution volumes with that of standard proteins (bovine serum albumin, myoglobin and insulin) mol.wts. of about 18000 and 9000 were estimated for the polypeptides in fractions II and III. Fraction I was eluted in the void volume, indicating a mol.wt. of around 30000 or greater. Fraction II had a markedly higher absorbancy ratio 254/280nm than had fractions I and III.

Chromatography of native enzyme on a similar column in 50mM-potassium phosphate (pH 7.5) is also shown in Fig. 3. In the absence of guanidine hydrochloride the enzyme was eluted as a single peak in the void volume.

Amino acid composition

Table 1 provides a summary of the results obtained by analysis of the amino acid compositions of the purified intact enzyme and bands I, II and III extracted from SDS/polyacrylamide gels (Fig. 2a). The composition data are calculated on the basis of the following apparent molecular weights: native enzyme, 54000 (Wallin *et al.*, 1978); band I, 27000; band II, 18000; band III, 8000–9000.

As shown in Table 1, the sum of the individual amino acids (residues/mol) for proteins corresponding to bands I, II and III is almost identical with the value for the corresponding amino acids in the native enzyme. Unfortunately, values for glycine in protein extracted from SDS/polyacrylamide gels could not be estimated because of contamination from the Tris/glycine buffer. The values for total amino acid residues/mol are the sums minus glycine (Table 1). However, because the composition of the native enzyme within the limits of experimental error equals twice that of band I, band I can be estimated to contain 17–18 residues of glycine. Consequently, the sum of the residues of glycine in bands II and III must add up to this value. The sum of serine residues in bands II and III shows the greatest deviation from the total number of serine residues in band I (+2 residues). Serine is one of the amino acids that is unstable to hydrolysis and consequently more difficult to estimate accurately.

By SDS/polyacrylamide-gel electrophoresis it was shown that band I yielded band II and band III upon

Fig. 3. Gel filtration in guanidine hydrochloride

Purified NAD(P)H dehydrogenase (50 μ g) was applied to a column (0.7cm $\times$ 33cm) of Sephadex G-75 and eluted in 50mM-potassium phosphate buffer (pH 7.5) containing 6M-guanidine hydrochloride. Absorbance of eluate was monitored at 280nm (—) and at 254nm (---) with a Uvicord III 2098 u.v.-absorptiometer. The flow rate was 12.6 ml/h. NAD(P)H dehydrogenase (50 μ g) was applied to an identical column in 50mM-potassium phosphate (pH 7.5) without guanidine hydrochloride and the absorption of the eluate monitored at 280nm (●). V_0 , Void volume.

Table 1. *Amino acid composition of NAD(P)H dehydrogenase and its subunits*

For NAD(P)H dehydrogenase, samples were hydrolysed for 24, 48 and 72h. Values for serine and threonine were obtained by extrapolation to zero time. Tryptophan was determined after hydrolysis at 105°C *in vacuo* for 22h in 4M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl)indole. Half-cystine was determined as cysteic acid after performate oxidation. For the subunits, bands I, II and III were isolated from SDS/polyacrylamide gels as explained in the text. The amino acid compositions are based on hydrolysis *in vacuo* for 24h with or without prior performate oxidation. Residues/mol were calculated on the basis of the apparent molecular weights: NAD(P)H dehydrogenase, 54000; band I, 27000; band II, 18000; band III, 8000–9000; Molecular weights are rounded off to the nearest 100.

Amino acid residue	Residues/mol of protein			
	NAD(P)H dehydrogenase	Band I	Band II	Band III
Asp*	37.4 (37)	18.6 (19)	13.6 (14)	5.8 (6)
Thr	21.8 (22)	9.5 (10)	6.5 (7)	2.9 (3)
Ser	35.4 (35)	17.6 (18)	13.1 (13)	6.8 (7)
Glu*	62.4 (62)	31.0 (31)	22.1 (22)	9.8 (10)
Pro	25.6 (26)	13.0 (13)	9.2 (9)	3.5 (4)
Gly	34.9 (35)	37.9†	43.8†	26.9†
Ala	38.8 (39)	18.7 (19)	13.4 (13)	5.0 (5)
Cys	5.7 (6)	2.7 (3)	2.6 (3)	0.2 (0)
Val	32.9 (33)	16.1 (16)	13.2 (13)	2.9 (3)
Met	7.8 (8)	4.2 (4)	3.4 (3)	1.0 (1)
Ile	20.7 (21)	10.4 (10)	7.8 (8)	2.1 (2)
Leu	47.2 (47)	22.7 (23)	17.7 (18)	4.0 (4)
Tyr	17.8 (18)	9.4 (9)	5.7 (6)	2.8 (3)
Phe	28.4 (28)	14.2 (14)	9.0 (9)	5.3 (5)
His	12.2 (12)	5.9 (6)	4.4 (4)	2.3 (2)
Lys	38.5 (38)	18.6 (19)	15.1 (15)	3.2 (3)
Trp	2.2 (2)	(1)‡	(1)‡	(0)‡
Arg	17.2 (17)	8.3 (8)	5.7 (6)	2.2 (2)
Total residues	486	223§	164§	60§
Mol.wt. of protein	54 100			
Mol.wt. of prosthetic group (FAD)	1600			
Mol.wt. of NAD(P)H dehydrogenase	55 700			

* Including asparagine and glutamine respectively.

† Values are too high because of glycine contamination (see the text).

‡ Tryptophan was shown by its absorption at 292nm to be present in bands I and II but not in band III.

§ Total residues minus glycine.

|| 2mol of FAD per mol of enzyme (54000).

re-electrophoresis (Fig. 2b). The yields of protein corresponding to bands II and III were calculated from the amino acid analysis to be 4.6 and 5.0nmol respectively, indicating stoichiometry.

Absorption characteristics of NAD(P)H dehydrogenase

The absorption spectrum of NAD(P)H dehydrogenase in 2.5mM-NH₄HCO₃ (pH 7.8) exhibits two absorption maxima (380nm and 450nm) and a shoulder (475nm), a pattern very similar to those of other flavin-containing dehydrogenases in the oxidized state (Williams, 1976) (Fig. 4).

The shoulder at 292nm indicates the presence of tryptophan in agreement with the amino acid analysis (Table 1).

The specific absorption coefficient at 271nm was calculated to be $A_{271}^{1\text{mg/ml}} = 36.8 \text{ litre} \cdot \text{g}^{-1} \cdot \text{cm}^{-1}$ with a 1cm light-path. The concentration of protein in the cuvette was determined by protein measurement and amino acid analysis, results from the two methods being in excellent agreement. On the basis of the absorption at 450nm, 2.3mol of FAD was estimated to be associated with 1mol of native enzyme (mol.wt. 54000).

Kinetic properties of the enzyme

The dependence of the specific activity of NAD(P)H dehydrogenase purified from Sprague-Dawley and Wistar rats on NADH concentration in Lineweaver-Burk plots showed the same K_m value

(74.1 μM) for both enzymes but V_{max} was different. V_{max} was 4673 $\mu\text{kat}/\text{mg}$ and 2967 $\mu\text{kat}/\text{mg}$ for the enzyme from Sprague-Dawley and Wistar rats respectively.

The effect of warfarin (Wallin *et al.*, 1978) on the velocity of the enzyme from Wistar rats was tested. Lineweaver-Burk plots in the presence and absence of this inhibitor revealed competitive inhibition with respect to NADH. From the ratio of the slopes the dissociation constant was calculated to be 6.7 μM at 22°C.

pH-dependence

Enzyme activity was measured in the pH range 6.2–10.2 by using three overlapping buffers (Fig. 5). The activity increased with increasing pH from 6.2

Fig. 4. Absorption spectrum of NAD(P)H dehydrogenase. The enzyme was dissolved (0.250 mg/ml) in 2.5 mM- NH_4HCO_3 , pH 7.8, at 22°C. The light-path was 1 cm.

Fig. 5. Effect of pH on the activity of purified NAD(P)H dehydrogenase

Enzyme purified from Wistar rats (125 ng) was added to the test system containing one of the following buffers: ●, 0.1 M-sodium phosphate; ○, 0.1 M-glycine/NaOH; ▲, 0.1 M-Tris/HCl. The buffers were adjusted with dilute HCl or NaOH to give the desired pH at 22°C. The activity was measured at 22°C in the presence of 1 mM-NADH.

to about 8.0 and showed a broad maximum between 8.0 and 9.0. At pH values above 10.2 or below 5.0 the enzyme was inactive. In 0.1 M-sodium acetate buffer (pH 4.0) the enzyme was irreversibly inactivated.

To detect an active and an inactive form of NAD(P)H dehydrogenase the enzyme was subjected to polyacrylamide-gel electrophoresis at pH 9.4 and 4.3. At pH 9.4 (active form) the enzyme migrated as a single band (Fig. 6) but at pH 4.3 (inactive form) several (at least five) discrete bands were seen.

Inactivation by side-chain-reactive reagents

The thiol-specific reagents HgCl_2 and 5,5'-dithiobis-(2-nitrobenzoic acid) effectively inactivated the enzyme, indicating that the activity is dependent on accessible thiol group(s). 5,5'-Dithiobis-(2-nitrobenzoic acid) at concentrations of 1 and 10 mM inhibited the enzyme activity by 67 and 100% respectively and HgCl_2 at concentrations of 0.2 and 1 mM inhibited it by 94 and 100%. In preliminary experiments guanidino-group-selective reagents (2,3-butanedione and 1,2-cyclohexanedione) and amino-group-selective reagents (2,4,6-trinitrobenzenesulphonic acid and pyri-

Fig. 6. Analytical polyacrylamide-gel electrophoresis of NAD(P)H dehydrogenase

Polyacrylamide-gel electrophoresis was carried out as described in the text at pH 9.4 with 15 μg of enzyme.

doxal 5'-phosphate) had a moderate inactivating effect. 2,3-Butanedione (10mM) inhibited the activity by 36%, whereas 1,2-cyclohexanedione at the same concentration inhibited it by only 19%. 2,4,6-Trinitrobenzenesulphonic acid (10mM) and pyridoxal 5'-phosphate (25mM) inhibited the enzyme activity by about 50%, suggesting some involvement of free amino group(s).

Immunology

The neutralizing effect of NAD(P)H dehydrogenase antiserum on enzyme activity was compared with that of normal rabbit serum. Antiserum (10 μ l) neutralized 220nkat of the enzyme activity. In immunodiffusion no cross-reaction was observed against methaemoglobin reductase in lysate of erythrocytes from rats (Hegesh *et al.*, 1971), showing that the erythrocyte 'diaphorase' is structurally different from NAD(P)H dehydrogenase (DT-diaphorase) in liver cytosol. Neither was any cross-reaction seen when antiserum was tested against supernatants from bovine or rabbit livers.

In immunoelectrophoresis against this antiserum, purified enzyme from Sprague-Dawley and Wistar rats each gave one precipitin line at the same position in the gel (Fig. 7). Identical results were obtained when liver supernatants from both rat strains were submitted to immunoelectrophoresis, showing the monospecificity of the rabbit antiserum. Immunodiffusion of liver supernatants and purified enzymes from Sprague-Dawley and Wistar rats against the same antiserum (raised against the Wistar enzyme) is shown in Fig. 8. Reactions of complete identity are seen between all precipitin lines.

Discussion

In a previous paper (Wallin *et al.*, 1978) a detailed description is given of the purification procedure for NAD(P)H dehydrogenase by affinity chromatography followed by adsorption chromatography on hydroxyapatite. This latter step has in the present work been replaced by DEAE-Sephadex chromatography to obtain a more convenient flow rate. No difference in yield or in the purity of the final product was observed.

The appearance of one protein band of mol.wt. 27000 when purified NAD(P)H dehydrogenase was subjected to SDS/polyacrylamide-gel electrophoresis suggested the existence of two subunits of similar molecular weight in the enzyme (Rase *et al.*, 1976; Wallin *et al.*, 1978). In our previous experiments the enzyme sample was not boiled in the presence of SDS before electrophoresis, a treatment that leads to the appearance of the 18000-mol.wt. band (band II) and the 8000-9000-mol.wt. band (band III) after SDS/polyacrylamide-gel electrophoresis. Re-electro-

Fig. 7. Immunoelectrophoresis of purified NAD(P)H dehydrogenase from Sprague-Dawley and Wistar rats. Electrophoresis was run for 60 min at 4°C and 10 V/cm in veronal buffer (pH 8.6). (a) Sprague-Dawley (0.8 μ g); (b) Wistar (0.9 μ g).

Fig. 8. Immunodiffusion of NAD(P)H dehydrogenase from Sprague-Dawley and Wistar rats

Wells 1 and 4: 100000g liver supernatant of Sprague-Dawley rats (25 mg/ml); well 2: 100000g liver supernatant of Wistar rats (26 mg/ml); wells 3 and 5: purified enzyme from Wistar rats (10 μ g/ml); well 6: purified enzyme from Sprague-Dawley rats (12 μ g/ml); centre well: rabbit antiserum against enzyme purified from Wistar rats.

phoresis of the 27000-mol.wt. band (band I) clearly demonstrated that the 18000-mol.wt. band and the 8000-9000-mol.wt. band are derived from the 27000-mol.wt. band and are not contaminants in the enzyme preparation.

Bands I, II and III did not stain for carbohydrate, indicating that NAD(P)H dehydrogenase is not a glycoprotein.

The content of hydrophobic amino acids in native NAD(P)H dehydrogenase does not differ much from the average of water-soluble proteins. NAD(P)H dehydrogenase is mainly found in the cytosol, but a small portion of activity is found associated with the microsomal fraction (Ernster, 1967). Repeated homogenization and washing of this fraction removed this activity (Wallin *et al.*, 1978), indicating that the enzyme is loosely associated with the membrane.

There was no significant difference in the distribution of hydrophobic residues in bands II and III to suggest which part might interact with the membrane.

Electrophoretic and amino acid analyses of the native and SDS-treated enzyme and the electrophoretic bands I–III suggest a model for NAD(P)H dehydrogenase consisting of two similar subunits of mol.wt. 27000, each subunit consisting of two polypeptide chains of mol.wt. 18000 and 8000–9000. The native enzyme (mol.wt. 55700) has two *N*-terminal amino acids (Asx and Glx) (Wallin *et al.*, 1978), which is consistent with the present model.

Complete separation of the two subunits of mol.wt. 27000 was obtained by polyacrylamide-gel electrophoresis in the presence of SDS because no protein band corresponding to a mol.wt. of about 54000 was seen on SDS/polyacrylamide gels after staining. However, neither SDS nor 6*M*-guanidine hydrochloride was able completely to dissociate the polypeptides of mol.wt. 18000 and 8000–9000 in the 27000-mol.wt. subunit. Because disulphide bridges were shown not to be involved, these results indicate that strong hydrophobic interactions exist between the two polypeptides within the 27000-mol.wt. subunit, a property different from the interaction between the two 27000-mol.wt. subunits. Fraction II (mol.wt. approx. 18000) from the Sephadex G-75 column exhibited a high absorbancy ratio 254/280 nm, indicating that FAD is bound to this polypeptide. FAD has an absorption maximum at 263 nm (Whitby, 1953). This observation fits very well with fluorescence measurements in extracts of bands I, II and III from SDS/polyacrylamide gels where FAD was detected in band I (mol.wt. 27000) and II (mol.wt. 18000), but not in band III (mol.wt. 8000–9000).

When estimated from the absorption at 450 nm, 2.3 mol of FAD were found to be associated with 1 mol of native enzyme. As previously reported (Wallin *et al.*, 1978), fluorescence measurements indicated a 1:1 stoichiometry. The presence of 2 mol of FAD/mol in the native enzyme fits well with the proposed model of two identical subunits in NAD(P)H dehydrogenase. Each 27000-mol.wt. subunit should consequently be associated with 1 mol of FAD, which is probably covalently bound to the apoenzyme in NAD(P)H dehydrogenase (Rase *et al.*, 1976). The lower value obtained by fluorescence measurements may be due to quenching.

NAD(P)H dehydrogenase from Wistar rats is active over a wide pH range with no sharp optimum, a different pattern from that of the bovine enzyme studied by Märki & Martius (1960), but which fits well with the results obtained by Ernster *et al.* (1962) for the enzyme purified from Sprague–Dawley rats.

The absorption spectrum for NAD(P)H dehydrogenase is quite characteristic for flavoproteins of the class with shoulders around 430 and 450 nm (Massey & Ganther, 1965). The structural basis for the differ-

ence between various classes of flavoproteins is presently unclear.

Lind *et al.* (1973) found a significantly higher activity of NAD(P)H dehydrogenase in Sprague–Dawley rats than in rats derived from the Wistar strain. Whether the difference reflected a difference in amount or activity of the enzyme was left unanswered. From Lineweaver–Burk plots the two enzymes had the same K_m value for NADH, but different V_{max} values. A K_m value of 74.1 μ M is in close agreement with results obtained by Ernster *et al.* (1972) for the enzyme from Sprague–Dawley rats (about 80 μ M). Immunodiffusion gave a reaction of complete identity between the two enzymes. The enzyme from Wistar rats therefore has no extra antigenic determinants and both enzymes showed the same electrophoretic mobility. NAD(P)H dehydrogenase isolated from Sprague–Dawley and Wistar rats are therefore two enzymes of great similarity. A systematic comparative study of their biochemistry would be necessary to detect the structural basis for the observed differences in specific activity.

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