CHAPTER V

DISCUSSION

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Solubilisation and Purification

NADase from ox brain was purified 800-1000 fold and the final specific activity (about 600 units/mg) obtained by this procedure is the highest so far reported for NADase from this source. The specific activities of other animal tissue NADases which have been highly purified are soluble bull semen enzyme (9906 units/mg) (Yuan and Anderson, 1971), the enzymes from pig brain (8100 units/mg) (Swislocki and Kaplan, 1967) and beef spleen (1150 units/mg) (Stathakos and Wallenfels, 1966a). A noteworthy feature of ox brain NADase is its high instability after a certain level of purification, which made it difficult to purify it further.

Windmueller and Kaplan (1962) attempted solubilisation of beef brain from its acetone powder by trypsin digestion which worked well with the pig spleen and pig brain enzymes. But they could not solubilize the beef brain enzyme using the conditions used for the enzymes from pig. Higher concentrations of trypsin resulted in destruction of the enzyme.

The success of elastase in effecting the solubilisation of beef brain and heart hexokinases and ox brain acetylcholinesterase suggested attempts at the solubilisation of brain NADase with elastase. Except for the enzyme from caudate nucleus acetone powder, elastase could not solubilize NADase from fresh tissue or acetone powder of whole or grey
or white matter of beef brain, fresh tissue and acetone powder of sheep brain or fresh tissue of beef spleen.

With the caudate nucleus acetone powder 20% of the activity of the particulate preparation was obtained in the 100,000 x g supernatant liquid. But since caudate nucleus constitutes a small portion of the brain, it was not a suitable source for further studies. The reason why the acetone powder of this part of the brain gave soluble enzyme with elastase is not clear and it may be worth investigating whether NADase from this source differs from that of other parts of brain.

Triton x 100 is a neutral detergent and was used to solubilize hexokinase from bovine brain and acetylcholinesterase from human caudate nucleus. Artman et al. (1964) had suggested that a detergent like deoxycholate could possibly be the agent of choice for solubilising NADase. They succeeded in bringing 69-75% of mouse liver microsomal NADase in solution. Triton x 100 was, therefore, tested and it effected 90 to 100% solubilisation of brain NADase at a concentration of 1.5%. But its presence was necessary to keep the enzyme soluble as shown by the experiments on removing the detergent by acetone or alcohol. Except for some purification by negative adsorption on DEAE-cellulose the enzyme could not be purified further by any of the several methods which were tested. However, a purity of 53 units/mg was obtained by DEAE-cellulose chromatography.
and it may be of interest in the light of the modified procedure used with the lipase solubilised enzyme to determine whether the Triton enzyme can be further purified by DEAE-cellulose chromatography.

While this work was in progress Swislocki et al. (1967) reported the success of porcine pancreatic lipase in solubilizing several mammalian NADases including that of beef brain. It is of interest that although isogamyl alcohol and DNA, trypsin and detergents have a limited effect in solubilising only some NADases, lipase was effective with all mammalian NADases indicating the possible involvement of lipids in the attachment of the enzyme to the particulate material.

Purification of NADase offered difficulty because of the high instability of the enzyme beyond a specific activity of about 30 units/mg protein, especially in low salt concentrations. The enzyme could be adsorbed on calcium phosphate gel, CM-cellulose and IRC-50 (XE-64) resin and eluted with some purification, but losses due to the instability of the enzyme were considerable. Though a final specific activity of 570 units/mg was obtained by procedure I, the yields were low and were only 1% of the soluble enzyme. The second procedure based on ammonium sulfate fractionation and DEAE-cellulose chromatography gave much better recovery of enzyme, with an average
purity of 240 units/mg, and a maximum of 575 units/mg and the yield was 16% of the soluble enzyme. Considerable difficulty was experienced in concentrating the enzyme, which was also highly unstable at this stage.

It is of interest that on acrylamide gel electrophoresis this preparation gave two bands, the major one containing over 85% of the total protein but no NADase activity. The enzyme band was clearly separated from the major protein impurity and contained too little protein for accurate measurement. These results suggest that it may be possible to use preparative gel electrophoresis to obtain NADase of high purity. In the case of the rat liver enzyme the final specific activity of 590 units/mg obtained was calculated for the polyacrylamide gel band by determining the protein by densitometer tracings. Swislocki and Kaplan (1967) observed that because of "the small amounts of this enzyme in tissues it has not heretofore been feasible to purify these preparations extensively with laboratory facilities which are commonly available for enzyme purification". They processed 200 pounds of pig brain and obtained a yield of 20 mg of purified enzyme.

Our observations agree with that of Quastel and Zatman (1953) that the white matter contains more activity than the grey matter.

One noteworthy and unexplained observation for the beef brain enzyme is that when the enzyme was extracted from
the acetone powder and fresh tissue, the acetone powder extract contained much higher activity than that from a corresponding amount of fresh tissue. 22 units of activity were obtained per g wet tissue with the 0.1M phosphate pH 6.8 extract and 125 units per g wet tissue from an equivalent amount of the acetone powder extract. The presence of a latent enzyme or incomplete extraction seems to be indicated or else an NADase inhibitor is present in the fresh tissue but not in the acetone powder extract.

Properties

On gel electrophoresis the ox brain enzyme moves towards the cathode (at pH 4.5). Swislocki et al. (1967) found that the crude enzyme on starch gel electrophoresis at pH 7.0 moved towards the cathode. The beef spleen, pig brain and spleen enzymes also moved towards the cathode, whereas the NADases from other tissues moved towards the anode at this pH.

Molecular weight

Swislocki et al. found by Andrews' method (Andrews, 1965) using Sephadex G-100 a molecular weight of 24,000 for beef brain NADase. The present studies gave a molecular weight of approximately 22,400 using the same method but with the purified preparation. The molecular weight of Triton x 100 solubilised NADase, using Andrews' method was found to be approximately 65,000. As indicated earlier
this value requires confirmation by a different method, preferably after further purification. This enzyme preparation undoubtedly aggregates to insoluble particles, presumably of larger molecular weight when Triton is removed from the enzyme. However, if the value of about 65,000 is confirmed for the Triton-solubilised preparation, it would indicate that the enzyme is bound to a fragment with twice its molecular weight presumably by an ester link and that the link with the larger molecule is broken by lipase treatment to give a soluble fragment with a molecular weight of about 22,400. Further work is needed to isolate the Triton solubilised and elastase solubilised enzymes and compare the composition, molecular weights and properties of these preparations.

**Stability**

The purified beef brain NADase was found to be unstable. In general purified mammalian NADases in the frozen state were found to be very stable, the exceptions being the rat liver enzyme (Bock et al. 1971) and the pig spleen enzyme (Dickerman et al. 1962). The rat liver enzyme (specific activity 590 units/mg) also became more thermolabile during purification and could be stabilised by adding serum albumin as in the case of the beef brain enzyme. The studies of Bock et al. showed that when assayed in the presence of albumin, enzyme activity increased by
about 25%. A similar observation was made with the beef brain enzyme. The purified pig spleen enzyme (specific activity 80 units/mg) lost 20% activity in 7 days at \(-20^\circ\). The purified beef brain NADase (600 units/mg) lost 20% activity in 24 hours. It is probably because of this instability and the fact that it is present in small amounts that the beef brain enzyme has not hitherto been purified to any significant extent. Of the various compounds tested only serum albumin imparted stability. This property of stabilization by serum albumin was probably due to its protein nature, since trypsin inhibitor could also bring about this stability and concentrated solutions of enzyme were less unstable than dilute solutions. This instability in dilute solutions is well known to be a general property of many enzymes. Further purification of the enzyme would require the use of methods such as ammonium sulfate fractionation in which concentrated enzyme solutions can be used.

Like other NADases of microbial and animal origin, beef brain NADase is stable over a wide pH range (pH 3.0 to 10.7). This stability appears to be a general property of NADases. The crude enzyme can be kept as long as 60 min at \(4^\circ\) at pH 3.0 without loss of activity and the purified enzyme as long as 30 min at pH 3.5 at \(4^\circ\).
Temperature stability

Purified NADase was stabler at lower temperatures and could be stored longer without inactivation at \(-20^\circ\) than at \(4^\circ\). There was no significant inactivation due to repeated freezing and thawing. Incubation of the enzyme without substrate (but with serum albumin) at pH 7.5 and \(4^\circ\) for 15 min. results in no loss of activity. It was not feasible to test NAD for its effect on stabilization in long term experiments owing to its rapid hydrolysis. Combinations of NAD with NA and INH were, however, ineffective in stabilizing the enzyme.

pH optimum

Like other NADases the beef brain enzyme is active over a wide pH range. The optimum pH for the beef brain NADase at 7.4 is not very sharp, the activity at pH 6.2 and 5.4 being 70% of that at pH 7.4 and 60% at pH 4.4 and 8.5. This pH profile is different from that for the beef spleen and beef lymph node enzymes (Stathakos and Wallenfels, 1966b). In the case of these enzymes the activity falls off sharply at pH 6.5 and is about 10% of the maximum at pH 4.0. The pH profiles of the enzymes from beef brain and pig brain (Swislocki and Kaplan, 1967) are similar.

Effect of substrate concentration on enzyme activity

The Km determined at different pHs for NADase showed no marked alteration between pH 8.2 and 4.0 indicating no active group involved at the active site ionizing in this
pH range. If a histidyl group were to be involved as suggested by Alivisatos (1959) it would have to be in unionized form. Ionizable histidine residue has a pK value of 6.0 (Mahler and Cordes, 1968). Yuan/Anderson (1972) found in the case of the bull semen enzyme and Apitz et al. (1971) for the pig brain enzyme also that the Km for NAD was not found to change with pH.

The Michaelis constant for beef brain NADase was of the order of $6 \times 10^{-5}$M which is similar to that of most mammalian NADases. For NADP the Kms at pH 7.5 and 4.0 were $2.7 \times 10^{-5}$M and $1.6 \times 10^{-5}$M respectively. NADP has a slightly greater affinity for the enzyme molecule than NAD. The additional (PO$_4^{3-}$) in NADP apparently does not alter the change of Km with pH.

**Inhibitors**

As with most mammalian NADases the Ki for the beef brain enzyme for nicotinamide was about 1 mM and the inhibition was of the noncompetitive type. InH showed competitive inhibition, the Ki being about $10^{-4}$M, which is similar to that of NADases of ruminants. The beef and lamb spleen and brain enzymes are powerfully inhibited by InH, the brain enzymes being slightly more strongly inhibited than the spleen enzyme. In the case of some non-ruminants (rat spleen, rabbit spleen and brain, horse spleen and brain, pig spleen and brain and frog spleen)
the activity is slightly (20 - 40%) inhibited by $10^{-2}$M INH or not inhibited at all (mouse spleen and brain, human prostate) (Zatman et al. 1954a).

Theophylline was found to be an inhibitor of the rabbit erythrocyte enzyme, the Ki for it being $2.5 \times 10^{-3}$M (Alivisatos et al. 1956). At $10^{-2}$M theophylline gives 30% inhibition with the beef brain enzyme. The inhibition by NA and theophylline did not alter with pH and was nearly the same at pH 7.5 and pH 5.0.

The recent observation that NADases of some sources can be inhibited by the substrate at alkaline pH but not at pH 6.0 (Green and Dobrjansky, 1971b) suggested a study of brain NADase. No inhibition was observed either at pH 6.0 or 8.0 by NAD. Neither did 6M urea inactivate the enzyme. The fact that its molecular weight was of the order of 22,400 shows that this enzyme belongs neither to class I nor class II according to the classification of Green and Dobrjansky (Chapter I, Section 3).

At $10^{-2}$M thymine inhibited the enzyme by 23%; a similar effect of thymine was also observed for the rabbit erythrocyte enzyme (Alivisatos et al. 1956). The inhibition by nicotinic acid, isonicotinic acid, AMP and ATP was negligible (10% at $10^{-2}$M). The powerful inhibitory effect of adenosine compounds observed for some of the NADases was not observed with beef brain NADase.
No SH group is involved in the activity of the enzyme, since none of the sulfhydryl agents (cysteine, thioethanol) or SH reactive agent (N-ethyl maleimide, p-CMB) had any effect on beef brain NADase. This observation is the same as that of other workers with other mammalian NADases.

Histamine was shown to inhibit the rat spleen enzyme (Devi and Mukundaw, 1963), but it did not inhibit ox brain NADase. Some other amino acids tested did not also inhibit this enzyme. Succinate and oxalate (0.06M) were shown to inhibit the rabbit erythrocyte enzyme (Sarkar, 1968), but they did not affect the beef brain enzyme at 10^-2M. A number of other intermediates of carbohydrate metabolism, glycolysis and citric acid cycle were without effect at 10^-2M to 10^-3M. Active group reagents like pyridoxal phosphate (reacting with lysine), sodium metabisulfite (reacting with carbonyl or S-S groups), diisopropylfluorophosphate (reacting with histidine and serine residues), semicarbazide and hydrazine (reacting with carbonyl groups) and sodium borohydride (-S-S group) did not affect NADase activity at 10^-2M to 10^-4M.

The enzyme has no/metal requirement. Metal chelating agents (EDTA and α,α'-dipyridyl) have no effect on its activity. There is no evidence in the literature for a metal or other cofactor requirement for this enzyme.
Metal or other cofactor requirements reported for the guinea pig lung NADase (Middleton and Devi, 1962) and pigeon liver NADase (Lubochinsky, 1958) require confirmation and demonstration that they are true NAD glycohydrolases.

Various salts, ZnSO₄, NaCl, KCl, MgCl₂, (NH₄)₂SO₄, NH₄Cl, sodium pyrophosphate at 10⁻³ to 10⁻¹M had no effect on the activity of beef brain NADase.

The effect of some nucleotides (α and β thiouridine, UDPG and cyclic AMP), hormones (tri-iodothyronine and thyroxine), vitamin (thiamine) and Yaminobutyric acid (important in brain metabolism) was tested but none of them had any effect on the activity of NADase.

Many of these compounds were also tested for their effect on NA inhibition but no modification of this inhibition was observed with any of them. Hence these compounds affect neither the enzyme nor its inhibition by NA.

A single enzyme is involved which acts both on NAD and NADP. This observation confirms previous data on ruminant NADases. For purified enzyme the NADase activity/NADPase activity ratio is about 2.0. This ratio is different from the ratio of 1.1 obtained by Swislocki et al. (1967) for the enzyme from beef brain. However, the ratio for crude elastase and lipase solubilized enzyme was about 1.5. The reason for this lower ratio was not further investigated. The NADase/NADPase ratio for the crude and purified enzymes has been
found to be different in the case of the ascites enzyme (Roemer et al. 1968; Green and Bodansky, 1964) and the pig brain enzyme (Apitz et al. 1971; Windmueller and Kaplan, 1962). The crude enzyme had a ratio of about 1.0 and the purified enzyme of 2.0.

The transglycosidase activity was not tested since this reaction has been studied in detail for the various NADases.

It was observed that those properties of the crude enzyme or elastase and Triton "solubilised" enzymes which were studied were similar to those of the lipase solubilised enzyme (except for the NADase/NADPase ratio) suggesting that solubilisation with lipase does not alter the properties of the enzyme. Swislocki and Kaplan (1967) also observed in the case of the pig brain enzyme that the properties of trypsin solubilised and the lipase solubilised enzyme were similar.