Purification and Characterization of Nicotinamide Deamidase from Yeast*

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Nicotinamide deamidase (YNDase) has been purified from yeast through the use of a six-step procedure that includes molecular-sieve high performance liquid chromatography. The final preparation was homogeneous by the criteria of sodium dodecyl sulfate-gel electrophoresis, and the enzyme specific activity was determined to be 175 μmol of nicotinate formed per min/mg enzyme. Gel electrophoresis and molecular-sieve high performance liquid chromatography were employed also to characterize YNDAse as a monomeric protein with a molecular weight of 34,000. A $K_i$ value for nicotinamide of 33 μM was determined for the deamidase activity at pH 6, and a pH range for optimal stability of 6–8.5 was established for this enzyme. The YNDAse activity was also examined over a pH range at several substrate concentrations and both the log $V_{max}$ and log $V_{max}/K_m$ plots versus pH suggested that a protonated amino acid residue with an apparent $pK_a$ value of 7.8 was essential to this activity. During an in vitro assay of the YNDAse-catalyzed formation of nicotinate, ammonia was generated and detected chemically. Inhibition of the YNDAse activity by nicotinaldehyde suggested the presence of either an essential lysine (Schiff's base formation) or cysteine residue (thiohemiacetal intermediate) at the YNDAse active site. The relatively large value of the nicotinaldehyde inhibition constant ($K_i = 68 \text{ μM}$), the observation that this analogue is a noncompetitive inhibitor of nicotinate formation, and the fact that this inhibition can be rendered irreversible through incubation with sodium borohydride, indicates that a Schiff's base intermediate is more likely to occur upon incubation of YNDAse with nicotinaldehyde. However, YNDAse is inactivated completely and irreversibly by N-ethylmaleimide at pH 6, and the enzyme is protected against this modification by either nicotinate or nicotinamide. These results suggest that both nicotinate and nicotinamide bind to YNDAse, even though the enzymatic reaction is essentially irreversible, and that a cysteine residue may be present at the YNDAse active site.

Niacin-tryptophan metabolism has been under investigation for over 100 years and is known to be associated with several nutrition-related diseases states in man (1). Nicotinamide and nicotinate are equally effective in relieving the dietary deficiencies causing pellagra and Hartnup's disease in man, because of the presence of an efficient nicotinamide deamidase (EC 3.5.19; YNDAse) activity which allows the conversion of the amide substituent to a carboxyl group (Equation 1). The YNDAse-catalyzed reaction is essentially irreversible and has been detected in several mammalian (2, 3) and bacterial (4, 5) sources.

During our survey of the phosphoribosyltransferase (PRTase) activities of yeast (6), quinolinate PRTase, nicotinamide PRTase, and nicotinate PRTase activities all were detected. However, the HPLC assay procedures that were employed in this study also detected, and were complicated by, the presence of an efficient nicotinamide deamidase in these yeast extracts. This enzyme had been detected previously in yeast (7, 8), but had never been isolated. We have elected to purify this enzyme and to examine its properties as part of a continuing analysis of NAD metabolism in yeast (9). A preliminary account of these results has been presented (10).

\[ \text{Nicotinamide} \rightarrow \text{nicotinate} + \text{NH}_3 \]  

EXPERIMENTAL PROCEDURES

RESULTS

Purification of Nicotinamide Deamidase—As shown in Table I, a six-step procedure was required to purify YNDAse from a baker's yeast extract. As shown in Fig. 5 of the Miniprint, two gel electrophoresis procedures revealed this protein preparation to be homogeneous. To our knowledge, this marks the first time that a nicotinamide deamidase has been isolated completely from any source.

Molecular Weight Determination—The molecular weights of the eukaryotic deamidases, that have been purified partially, have been found, consistently to lie within the range of 150,000–300,000 (2, 3). In contrast to these results, the final

* The abbreviations used are: YNDAse, nicotinamide deamidase from yeast; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide; PRTase, phosphoribosyltransferase; SDS, sodium dodecyl sulfate (Miniprint); CHES, 2-(cyclohexylamino)ethanesulfonic acid (Miniprint); HTP, hydroxyapatite (Miniprint).

1 Portions of this paper (including "Experimental Procedures" and Figs. 1–7) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass.

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Summary of the purification of nicotinamide deamidase from yeast

<table>
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<th>Procedure</th>
<th>Volume</th>
<th>Concentration</th>
<th>Total mg</th>
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<th>Specific activity</th>
<th>Total units</th>
<th>Yield %</th>
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*To date 10 samples (20 μl each) have been injected onto the Bio-Sil TSK-250 column. The eluent that contained YNDase activity was concentrated to 1 ml. The remaining 3.6 ml of active YNDase from the phenyl-Sepharose column has been stored at -76 °C for 4 months and has retained its activity through this storage procedure.

Step of purification of the YNDase made use of an HPLC column that had been standardized with molecular weight markers and indicated that the activity eluted where much smaller proteins (M, = 30,000) eluant. This suggestion was confirmed with standardized nondenaturing and sodium deoxycholate-gel electrophoresis techniques (Fig. 5). It would appear therefore that the homogenous deamidase from yeast is similar to the bacterial enzymes in that it is a monomeric protein with a molecular weight of 34,000 ± 2,000. Our results do not rule out the possibility that this active protein may be part of an active oligomer which occurs in solution under different conditions. The amino acid composition of YNDase has been determined (Table II) and confirms the molecular weight of YNDase.

Kinetic Analysis—Values for Vmax and Ks for the YNDase catalyzed reaction, as determined from a double reciprocal plot of the velocity of nicotinate formation versus nicotinamide concentration, were determined over a pH range of 5–9 (Figs. 6 and 7 of the Miniprint). As shown in Fig. 7A, a significant loss of enzymatic activity was observed when YNDase was incubated for 10 min at pH values outside this pH range. A calculated Ks value of 33 ± 15 μM was determined for nicotinate at within the 5–9 pH range, whereas an optimal Vmax value equal to 1.58 ± 0.2 units (micromoles of nicotinate formed per min)/mg was calculated from assay solutions within a 5.5–7 pH range. Using a computer program written by R. S. Strauss in this laboratory, Dixon plots (Fig. 7, B and C) of log Vmax and log Vmax/Ks versus pH, based on Equation 2, were fit to theoretical curves, from which an apparent pKs value of 7.8 was obtained.

\[
V = \frac{(1 + [H^+]K_o + K'_o[H^+] + (K'_o[S] + 1 + [H^+]K_o + K'_o[H^+])}{V_{max}}
\]

These studies suggested to us that at least one essential protonated amino acid residue must be present at the YNDase active site. Thus, chemical modification analyses were initiated to see if this amino acid could be identified. In addition to the quantitative studies of YNDase catalysis, one qualitative experiment was performed. Through the use of standard laboratory procedures, ammonia was observed to be produced from a 30-min incubation of YNDase with nicotinamide, indicating that an amine-acceptor molecule is not required during the formation of nicotinate under these in vitro conditions.

Modification with Nicotinaldehyde—We postulated that a lysine residue might exist near to the nicotinamide binding site on YNDase that might form a tight ion-pair bond with the product carboxyl group subsequent to the formation of nicotinate. In order to test whether this residue might indeed appear at the enzyme active site, we chose to examine the time-dependent effects of nicotinaldehyde on YNDase activity. The effect of incubations of YNDase with nicotinaldehyde, prior to the assay of the enzyme on the rate of nicotinate formation, in the presence and absence of sodium borohydride, is shown in Fig. 8. As shown in Fig. 8A, there occurs a rapid loss of YNDase activity, the percentage of which is dependent on the nicotinaldehyde concentration, and after the initial loss of activity, no further loss is observed. Moreover, fairly high concentrations (20 mM) of nicotinamide will effectively protect the enzyme from inactivation by 2 mM reagent (Fig. 8A), although an equilibrium activity is still maintained during the time course of this incubation. These results suggest the reversible formation of a thiohemiacetal (15), a diol (16), or a Schiff's base intermediate (17) between the enzyme and the aldehyde group of this substrate analogue.

Volfenden (21) and others (18) have demonstrated that aldehydic substrate analogues can bind tightly and reversibly at enzyme active sites where cysteine or serine residues are known to form covalent intermediates with amide-containing substrates. We therefore elected to examine the kinetic effects of nicotinaldehyde on the YNDase-catalyzed nicotinate formation. Interestingly, as shown in Fig 9, A and B, nicotinaldehyde is an apparent noncompetitive inhibitor of nicotinate
formation, and is defined by an inhibition constant (68 μM) that is slightly larger than the $K_{\text{m}}$ of nicotinamide (33 μM).

Thus, the interaction between nicotinaldehyde and YNDase is most likely not through a thiohemiacetal or gem-diol intermediate. In contrast, the formation of a Schiff's base intermediate was indicated by a flow dialysis experiment (Fig. 1B), during which the YNDase activity was nearly all recovered unless the incubation had been performed in the presence of the reducing agent sodium borohydride. In a control experiment, it was shown that this irreversible loss of enzymatic activity was not due to the addition of the borohydride since during which the YNDase activity was nearly all recovered.

Figure 8. Incubations of nicotinamide deamidase with nicotinaldehyde. A, incubations in the presence of: a, 2 mM nicotinaldehyde and 20 mM nicotinamide; b, 2 mM nicotinamide; and c, 10 mM nicotinaldehyde. B, reactivation of the enzyme through flow dialysis, subsequent to incubations with: a, 10 mM nicotinaldehyde; b, 10 mM nicotinaldehyde plus 20 mM sodium borohydride. Assay conditions were as described in the legend to Fig. 1.

Figure 9. Kinetic analysis of the effect of nicotinaldehyde on the YNDase catalyzed formation of nicotinate. A, double reciprocal plot of the initial velocity (micromoles of nicotinate formed per min) versus nicotinamide concentration, and the following series of nicotinaldehyde concentrations: no addition (bottom line); 16.5 μM, 33 μM, and 49.6 μM (top line). B, plot of the reciprocal velocity versus nicotinaldehyde concentration. The concentrations of nicotinamide employed were: 200 μM (bottom line), 100 μM, 50 μM, and 20 μM (top line). Assay conditions were as described in the legend to Fig. 1.

The properties of YNDase most closely approximate those of the bacterial enzyme isolated by Tanigawa et al. (5, 20). The pH optimum for catalytic activity (pH 5–7) is the same and the two active proteins are approximately the same size ($M_r = 30,000–50,000$). In addition, the $K_{\text{m}}$ values for nicotinamide determined for the yeast (33 μM) and bacterial (200 μM) NDases are consistent with the formation of a relatively tight enzyme-substrate complex. In contrast, all of these results differ with those obtained for the mammalian enzymes that have been examined to date (2, 3). The deamidase contained in mouse neuroblastoma cells is a tetrameric glycoprotein ($M_r = 65,000$) subunits. The nicotinamide $K_{\text{m}}$ value for this enzyme is 1 mM and optimal activity is achieved at pH 7. Moreover, NDase purified partially from rat liver also is an oligomeric protein composed of large subunits, but this enzyme has an even lower affinity for nicotinamide and an apparent optimal pH in the alkaline range. Interestingly, the apparent optimal pH for rat liver NDase changes as the enzyme is carried through a purification procedure (3). Thus, we believe that these mammalian NDase activities must be isolated more completely before any further comparisons with the yeast enzyme are made.

Wolfenden and his colleagues (Ref. 21 and 22, and references therein) have demonstrated that active-site directed aldehydic reagents can serve as potent reversible inhibitors and transition state analogues for a variety of enzymatic reactions that involve the breaking of amide or peptide linkages, including the papain (23), L-asparaginase (16), and elastase (24)-catalyzed reactions. Since there are similarities between the papain-catalyzed breaking of a peptide bond (apparently involving the formation of an acyl-enzyme intermediate with a cysteine residue (23)) and the asparaginase-catalyzed deamination of asparagine (perhaps involving a cyclic tetrahedral intermediate (16)), we elected to test whether nicotinaldehyde, a substrate analogue, was a potent inhibitor of YNDase activity. Our results suggest that nicotin-
aldehyde can bind to the YNDase active site as well as to other residues of the enzyme with a relatively weak affinity, and this interaction may not therefore reflect the transition state that appears during nicotinate formation. However, our chemical modification results suggest that lysine may be present at the YNDase active site. This residue would be modified by NEM. It has been suggested that other residues of the enzyme with a relatively weak affinity, bacterial glutaminase may proceed through an analogous double-displacement mechanism (25, 26).

Since bakers' yeast contains an efficient YNDase, this organism is able to make use of both niacinamide and niacin as nutrients to bring about the synthesis of NAD through the use of the Preiss-Handler pathway (27). This YNDase activity may be important in determining the relative concentrations of nicotinate mononucleotide and nicotinamide mononucleotide in the yeast cell, which in turn may define the physiological substrate for nicotinate/nicotinamide mononucleotide adenyltransferase in yeast (28). In addition however, this enzyme may be present to salvage the nicotinamide that results from the formation of ADP-ribose from NAD. It is now well known that other eukaryotic organisms use ADP-ribose and poly-ADP-ribosylated proteins as metabolic regulators, and that a quantity of nicotinamide results during ADP-ribose synthesis (29). We propose to search for ADP-ribosyltransferase (30) and NAD glycohydrolase (31) activities in bakers' yeast as the next step in our studies of NAD metabolism in these cells. The presence of these enzymes would indicate that yeast metabolism may also make use of ADP-ribose in cell regulation.

Acknowledgments—We acknowledge the contributions of Rosalyn S. Strauss, who helped with the interpretation of the YNDase pH dependence and Linda Z. Ali, who participated in the design of the HPLC assay procedure.

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Nicotinamide Deamidase in Yeast

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Experimental Procedures

Material. Nicotinamide, nicotinic acid, diethanolamine, metaphosphoric acid, oxamide, and the posing compound used in the nicotinamide deamidase were obtained from Sigma (St. Louis), whereas Bacto-agar was obtained from Difco Laboratories (Detroit, MI). Other chemicals and reagents were purchased from Fisher Scientific. The biospecificity analysis was performed with an evaporative light-scattering detector (ELSD) and a Waters 2487 dual wavelength detector. The reaction mixture was maintained at 30 °C.

Assay Procedures. A microplate high-pressure liquid chromatography (HPLC) procedure (1.10) was used to assay the deamidase activity of the yeast under the following conditions: 0.20 ml of yeast extract and 0.04 ml of 0.5 M potassium phosphate buffer (pH 7.0) were mixed and incubated at 30 °C for 30 min. The reaction was stopped by the addition of 0.2 M acetic acid. The reaction mixture was then centrifuged at 10,000 × g for 5 min. The supernatant was transferred to a 10-ml vial and the absorbance at 260 nm was recorded. The absorbance of the samples was measured against a distilled water blank.

Results

Figure 1. Profiles of the elution of YNClass through Phenyl-Sepharose CL-4B and DEAE-Sepharose. A. Immunoabsorbent gave a peak activity at 260 nm (fraction 2). The elution profile was monitored by measuring the absorbance at 260 nm (fraction 2). The standard HPLC assay procedure, as described in the legend to Figure 1, was employed to monitor the YNClass activity. In these fractions, Sucrose 200 mg volumes were dialyzed in 0.1 M potassium phosphate buffer (pH 7.0) for 30 min. The absorbance of each fraction was measured against a distilled water blank. The dashed line represents the estimation of the protein concentration by absorption at 280 nm. The dashed line represents the estimation of the protein concentration by absorption at 280 nm.

Discussion

The final step in this purification procedure was HPLC gel-filtration chromatography. A Bio-Rad TSK-250 column was packed with 5 mg of the protein extract on the column and equilibrated with a mobile phase comprised of 0.05 M sodium acetate buffer (pH 5.0, 0.2 M sodium phosphate buffer (pH 5.0). The peak obtained was collected and concentrated in a microtiter cell. The YNClass activity (A) at each fraction was determined with the HPLC assay procedure described in Figure 1, whereas the protein concentration in these fractions was estimated by measuring their absorbance at 280 nm. The dashed line represents the estimation of the protein concentration by absorption at 280 nm.

Conclusions

The biospecificity analysis was performed with an ELSD and a Waters 2487 dual wavelength detector. The reaction mixture was maintained at 30 °C.

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Figure 4. Molecular weight determinations of YNDase. A: A plot of the molecular weights of standard proteins (egg albumin 45,000, myoglobin 19,000, beta-lactoglobulin 34,000, and lysozyme 14,300) versus their elution volume (from 10). B: A plot of the molecular weights of standard proteins (egg albumin 45,000, myoglobin 19,000, beta-lactoglobulin 34,000, and lysozyme 14,300) versus their elution volume (from 10). The conditions of the electrophoresis on the polyacrylamide gel are as described in "Experimental Procedures."

Figure 5. Double reciprocal plots of the initial velocity of niacinamide formation from the addition of 10 μM NAD and various concentrations of YNDase activity versus reaction time. The plot was performed at pH 6.5. The assay conditions were as described in Figure 1.