Discovery of a Third Coenzyme in Sarcosine Oxidase†

Annie Willie and Marilyn Schuman Jorns*

Department of Biochemistry, Medical College of Pennsylvania and Hahnemann University, Philadelphia, Pennsylvania 19102

Received October 4, 1995⊗

ABSTRACT: Denaturation of recombinant sarcosine oxidase or the natural enzyme isolated from *Corynebacterium* sp. P-1 with guanidine hydrochloride releases noncovalently bound FAD and a second UV-absorbing component (peak 2) which comigrates with NAD^+ during reversed-phase HPLC. Both FAD and peak 2 are also found in extracts prepared by incubating sarcosine oxidase at 37 °C for 30 min, a procedure which causes partial (~50%) release of the enzyme's noncovalently bound FAD. Peak 2 in the 37 °C extract is heat labile and decomposes upon boiling for 5 min at pH 8.0. A similar instability was observed with NAD^+. Reaction of the 37 °C extract from sarcosine oxidase with phosphodiesterase yields nicotinamide mononucleotide, AMP, and FMN, as expected for a mixture containing NAD^+ and FAD. Peak 2 was converted to NADH upon reaction of the 37 °C extract with yeast alcohol dehydrogenase in the presence of ethanol. Guanidine hydrochloride extracts, prepared from recombinant or natural enzyme, contain 1 mol of NAD^+/mol of FAD. Since sarcosine oxidase contains 1 mol of noncovalently bound FAD, the results show that the enzyme also contains 1 mol of NAD^+. The NAD^+ is tightly bound and is not lost during enzyme purification. It is not susceptible toward hydrolysis by NADase, reduction by alcohol dehydrogenase, or nucleophilic attack by cyanide. Unlike the flavins in sarcosine oxidase, NAD^+ is not reduced by sarcosine and is not in redox equilibrium with the flavins.

Sarcosine oxidase is produced as an inducible enzyme when *Corynebacterium* sp. P-1 is grown with sarcosine as source of carbon and energy. In the presence of O_2 and H_4-folate,[1] the enzyme catalyzes the oxidative demethylation of sarcosine to yield glycine, hydrogen peroxide, and 5,10-CH_2-H_4folate (eq 1). (Formaldehyde is generated in place

$$CH_3NH_2^+CH_2CO_2^- + O_2 + H_4\text{folate} \rightarrow H_3N^+CH_2CO_2^- + 5,10\text{-}CH_2\text{-}H_4\text{folate} + H_2O_2 \quad (1)$$

of 5,10-CH_2-H_4folate when sarcosine is oxidized in the absence of H_4folate.) Corynebacterial sarcosine oxidase contains four different subunits ($\alpha\beta\gamma\delta$), a single binding site for sarcosine, and both covalently and noncovalently bound flavins. The noncovalent flavin accepts electrons from sarcosine which are then transferred in one-electrons steps to the covalent flavin where oxygen is reduced to hydrogen peroxide (Kvalnes-Krick & Jorns, 1986, 1987; Jorns, 1985; Zeller et al., 1989; Ali et al., 1991). The genes encoding the enzyme's four subunits have been cloned, sequenced, and overexpressed in *Escherichia coli* (Chlumsky et al., 1993; Chlumsky et al., 1995). Sequence analysis reveals homology of the corynebacterial sarcosine oxidase subunits with various other proteins including monomeric sarcosine oxidases, dimethylglycine dehydrogenase, T-proteins, and opine oxidoreductases.

In this paper, we report the discovery of a third coenzyme, NAD^+, that is present in stoichiometric amounts in both recombinant sarcosine oxidase and the natural enzyme isolated from *Corynebacterium* sp. P-1.

EXPERIMENTAL PROCEDURES

Materials. Yeast alcohol dehydrogenase, NADase, phosphodiesterase, ADP, AMP, adenosine, cyclic AMP, FAD, FMN, NAD, NADH, NMN, riboflavin, 5-CHO-H_4folate, 5-CH_3-H_4folate, sodium cyanide, semicarbazide, and charcoal were obtained from Sigma. H_4folate was obtained from Dr. B. Schirck's Laboratories. Guanidine hydrochloride was obtained from Heico Chemical, Inc. Microcon-3 concentrators were obtained from Amicon.

Enzyme Preparation. Recombinant sarcosine oxidase was isolated from an *E. coli* clone, XL1-Blue/pLJC305. The natural enzyme was obtained from *Corynebacterium* sp. P-1 cells grown in the presence of sarcosine. Enzyme purification was conducted similar to that previously described (Kvalnes-Krick & Jorns, 1986; Chlumsky et al., 1993). Protein concentration and enzyme activity were determined as described by Chlumsky et al. (1993).

Chromatography and Spectroscopy. The enzyme was completely denatured by treating with 3 M guanidine hydrochloride in 10 mM potassium phosphate buffer, pH 8.0. Small molecules released from the enzyme were recovered in the filtrate obtained after microfiltration (Microcon-3). A 37 °C extract was similarly prepared using enzyme that had

† This work was supported in part by Grant GM 31704 (M.S.J.) from the National Institutes of Health.

* To whom correspondence and requests for reprints should be addressed. Phone: (215) 762-7946. FAX: (215) 246-5836. E-Mail: jornsm@hal.hahnemann.edu.

⊗ Abstract published in *Advance ACS Abstracts,* December 15, 1995.

[1] Abbreviations: FAD, flavin adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; H_4folate, tetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; 5,10-CH^+-H_4folate, 5,10-methenyltetrahydrofolate; 5-CHO-H_4folate, 5-formyltetrahydrofolate; 10-CHO-H_4folate, 10-formyltetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; NAD^+, nicotinamide adenine dinucleotide; $NADP^+$, nicotinamide adenine dinucleotide 2′-phosphate; NMN, nicotinamide mononucleotide; NADH, 1,4-dihydronicotinamide adenine dinucleotide.

0006-2960/95/0434-16703$09.00/0

been incubated for 30 min at 37 °C in 10 mM potassium phosphate, pH 8.0, a procedure which causes partial denaturation (*vide infra*). HPLC analyses were conducted using a Rainin gradient HPLC system equipped with a Rainin Microsorb C_{18} reversed-phase column (5 μm, 46 mm × 250 mm). The elution profile (flow rate = 1.0 mL/min) was adapted from procedures previously described (Sato et al., 1993; DuPleiss et al., 1994): 5 min isocratic elution with 0.1 M potassium phosphate buffer, pH 5.3, 25 min linear gradient to 50% methanol, and 5 min linear gradient to 75% methanol. The column eluate was monitored by its absorbance at 260 nm. Absorption spectra were recorded at 25 °C using a Perkin-Elmer Lambda 3B spectrometer.

Reaction of Sarcosine Oxidase Extracts with Phosphodiesterase or Alcohol Dehydrogenase. Phosphodiesterase treatment of the 37 °C extract from sarcosine oxidase or various standard samples was conducted by incubation for 30 min at 25 °C with 0.002 unit of phosphodiesterase. Alcohol dehydrogenase reactions were conducted as specified by Klingenberg (1985) and are described briefly in the legend to Figure 3.

Incubation of Native Sarcosine Oxidase with Charcoal, NADase, or Alcohol Dehydrogenase. Sarcosine oxidase (19 μM) was incubated with charcoal (5 mg/mL) in 10 mM potassium phosphate buffer, pH 8.0 at 25 °C, for 16 h. The charcoal was removed by centrifugation. The supernatant was used to prepare a guanidine hydrochloride enzyme extract, as described above. The ratio [FAD]/[NAD^+] in the guanidine hydrochloride extract was determined by HPLC analysis as described in the legend to Table 2. The absolute amounts of FAD and NAD^+ in charcoal-treated enzyme were estimated by comparison with HPLC data obtained with an extract prepared from an otherwise identical control sample. For incubations with NADase, sarcosine oxidase (12 μM) or a control sample of free NAD^+ (10 μM) was incubated for 16 h with 1.6 units of NADase in 10 mM potassium phosphate buffer, pH 7.3 at 25 °C. The extent of NAD^+ hydrolysis was determined by HPLC analysis of the control sample or a guanidine hydrochloride extract of the enzyme sample. Reaction of sarcosine oxidase (18 μM) with alcohol dehydrogenase (1.0 unit) was conducted in 50 mM pyrophosphate buffer, pH 8.8, containing 0.6% ethanol and 23 mM semicarbazide at various temperatures, as described in the Results section.

Attempts To Reduce NAD^+ in Sarcosine Oxidase Using Sarcosine Oxidation Products and Various Folate Derivatives. 10-CHO-H_4folate and 5,10-CH^+-H_4folate were prepared from 5-CHO-H_4folate according to the methods of Rabinowitz (1963). 5,10-CH_2-H_4folate was prepared by mixing 1 mM formaldehyde and 0.2 mM H_4folate in 0.2 M Tris-HCl buffer, pH 7.4, under an argon atmosphere in a procedure similar to that previously described (Ljungdahl et al., 1980). Incubations were performed with 3−10 μM sarcosine oxidase using a specially constructed cuvette which was made anaerobic as described previously (Jorns & Hersh, 1975). Incubations with 0.1 mM glycine, 0.1 mM formaldehyde, or 0.5 mM 5-CHO-H_4folate were conducted in 10 mM potassium phosphate buffer, pH 8.0 at 25 °C. Incubations with 0.2 mM 5,10-CH_2-H_4folate were performed in 0.2 mM Tris-HCl buffer, pH 7.4. Incubations with 0.02 mM 10-formyl-H_4folate or 1 mM 5-CH_3-H_4folate were in 50 mM Tris-HCl buffer, pH 8.0. The reactions were followed for a minimum of 6 h by periodically recording absorption spectra (300−550 nm).

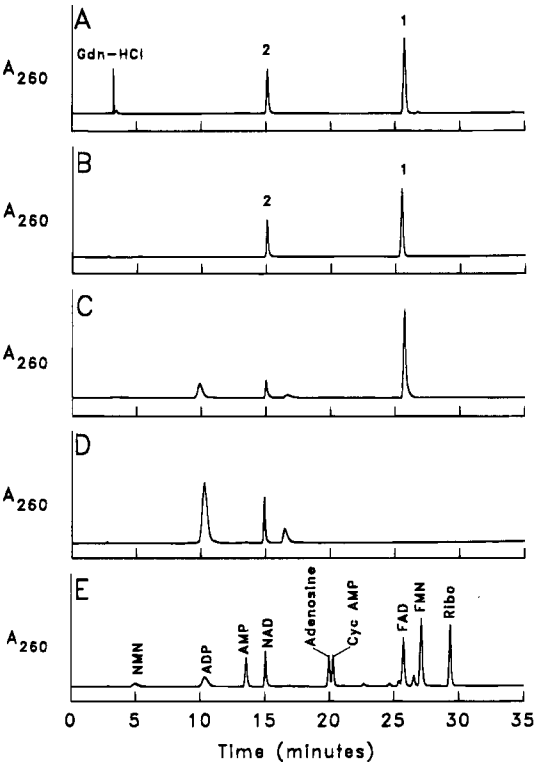

FIGURE 1: HPLC analysis of extracts prepared by treating recombinant sarcosine oxidase under various conditions. Extracts were prepared by denaturing the enzyme with 3 M guanidine hydrochloride (panel A) or by a 30 min incubation at 37 °C in 10 mM potassium phosphate buffer, pH 8.0 (panel B). The elution profile in panel C was obtained when the 37 °C extract was heated at 100 °C for 5 min prior to analysis. Panel D was obtained after a solution of NAD^+ was boiled in the same buffer for 5 min. Panel E shows the profile obtained for a set of standards, including untreated NAD^+.

RESULTS

Identification of NAD^+ in Sarcosine Oxidase Extracts. Denaturation of recombinant sarcosine oxidase with 3 M guanidine hydrochloride results in complete loss of enzyme activity and the quantitative release of the enzyme's noncovalent flavin. The extract obtained after microfiltration was analyzed by reversed-phase HPLC. The elution profile obtained by monitoring absorbance at 260 nm (Figure 1A) exhibits an expected peak for FAD (peak 1, 25.2 min) plus a second UV-absorbing component (peak 2, 14.8 min). A third peak eluting at 3 min is due to guanidine hydrochloride. Both FAD and peak 2 are also found in extracts prepared by incubating recombinant sarcosine oxidase at 37 °C (Figure 1B), a procedure which causes partial (~50%) release of the enzyme's noncovalent flavin. Similar results were obtained for extracts prepared using the natural enzyme isolated from *Corynebacterium* sp. P-1 (data not shown).

The noncovalent flavin is completely released by boiling sarcosine oxidase for a few minutes, but this denaturation method was avoided because significant decomposition of peak 2 was observed after boiling the 37 °C extract for 5 min (Figure 1C). Peak 2 comigrates with NAD^+, as judged by comparison with the elution profile obtained for a set of standards (Figure 1E). Like peak 2, NAD^+ also decomposed

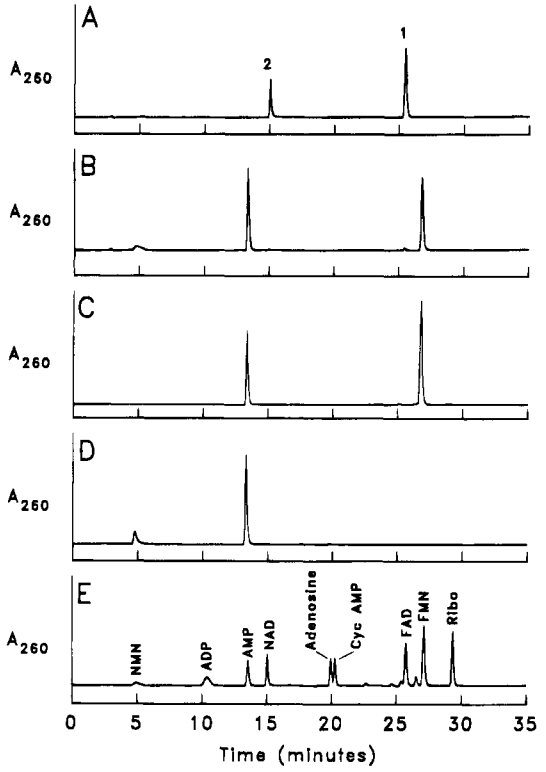

FIGURE 2: Reaction of phosphodiesterase with the extract prepared by incubating recombinant sarcosine oxidase at 37 °C for 30 min. The elution profiles obtained for the 37 °C heat extract before and after phosphodiesterase treatment are shown in panels A and B, respectively. The chromatograms obtained after FAD or NAD^+ was treated with phosphodiesterase are shown in panels C and D, respectively. For comparison, the set of standards shown in panel E includes untreated NAD^+ and FAD.

when boiled for 5 min in the same phosphate buffer (Figure 1D). The NAD^+ decomposition product eluting at 10.2 min was identified as ADP by comparison with standards and by its conversion to AMP upon treatment with phosphodiesterase. The slower eluting decomposition product (16.4 min) coeluted with nicotinamide (data not shown). Analogous decomposition products have been observed after boiling $NADP^+$ (Bernofsky, 1980). Lowry et al. (1961) found that NAD^+ decomposition at 100 °C was markedly accelerated by phosphate buffer.

The elution profiles obtained before and after phosphodiesterase treatment of the 37 °C extract from recombinant sarcosine oxidase are shown in panels A and B of Figure 2, respectively. Panels C and D of Figure 2 show chromatograms obtained after treatment of FAD and NAD^+, respectively, with phosphodiesterase. The products of the phosphodiesterase reaction with the 37 °C extract (NMN, AMP, and FMN) were identified by comparison with a set of standards (Figure 2E) and are those expected for a sample containing both NAD^+ and FAD. Similar results were obtained with extract prepared from the natural enzyme (data not shown).

Figure 3 shows the spectral changes observed when the 37 °C extract from recombinant sarcosine oxidase was reacted with alcohol dehydrogenase in the presence of added ethanol. Alcohol dehydrogenase caused a rapid increase in absorbance at 340 nm, accompanied by a decrease in absorbance at 260 nm. As shown in the inset, the difference spectrum is very similar to that expected upon conversion of NAD^+ to NADH ($\Delta\epsilon_{340} = +6.3\ mM^{-1}\ cm^{-1}$; $\Delta\epsilon_{260} = -3.3\ mM^{-1}\ cm^{-1}$) (Beutler & Supp, 1983). Alcohol dehydrogenase did not affect the FAD in the extract, as judged by the absence of absorbance changes at 450 nm. The results unambiguously show that the second UV-absorbing component in the sarcosine oxidase extract must be NAD^+.

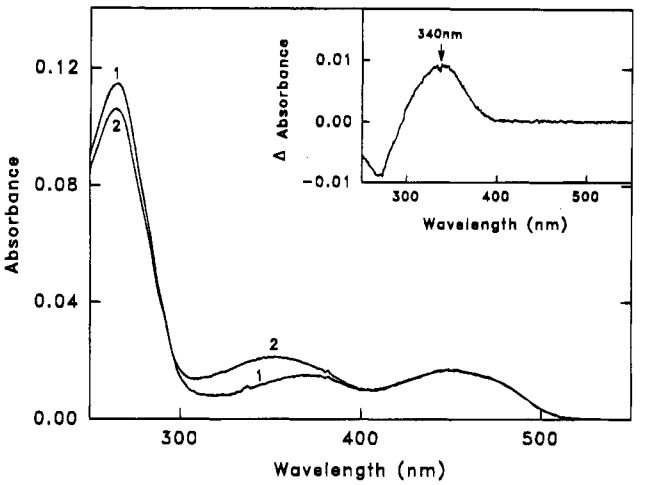

FIGURE 3: Effect of alcohol dehydrogenase on the absorption spectrum of the extract prepared by incubating recombinant sarcosine oxidase at 37 °C for 30 min. The enzyme extract in 10 mM potassium phosphate buffer, pH 8.0, was diluted 1:1 with 100 mM sodium pyrophosphate, pH 8.8, containing 45 mM semicarbazide. Curve 1 was recorded after addition of 0.6% ethanol. Curve 2 was recorded after reaction with yeast alcohol dehydrogenase (1.8 units/mL) for 5 min at 25 °C. The inset shows the difference spectrum obtained by subtracting curve 1 from curve 2.

Amount of NAD^+ in Recombinant and Natural Sarcosine Oxidase. The alcohol dehydrogenase assay could not be used with guanidine hydrochloride extracts. Although the enzyme is not completely denatured by the 37 °C treatment, preliminary studies with 37 °C extracts were conducted to develop a nonenzymic method for NAD^+ analysis which could be used with guanidine hydrochloride extracts. The NAD^+ concentration in various dilutions of a 37 °C extract from recombinant sarcosine oxidase was estimated on the basis of the absorbance of the extract at 260 nm (corrected for the contribution due to FAD) or the increase in absorbance at 340 nm in the alcohol dehydrogenase assay. Similar results were obtained using either method with the enzyme extract or with NAD^+ standard solutions (Table 1).

The total amount of NAD^+ in native enzyme was estimated using guanidine hydrochloride extracts prepared from recombinant or natural enzyme. These extracts were found to contain 1 mol of NAD^+/mol of FAD, based on the absorbance of the samples at 260 nm (NAD^+) and 450 nm (FAD). Similar results were obtained by HPLC analysis where the molar ratios were estimated on the basis of the areas under the FAD and NAD^+ peaks (Table 2). Since sarcosine oxidase contains 1 mol of noncovalently bound FAD, the enzyme must also contain 1 mol of NAD^+.

Properties of the NAD^+ in Sarcosine Oxidase. That NAD^+ is tightly bound to sarcosine oxidase is demonstrated by the stoichiometric amount of NAD^+ found in the isolated enzyme. Loss of NAD^+ (~20%) was observed following prolonged incubation with charcoal at 25 °C, but this was accompanied by a similar loss of the enzyme's noncovalent flavin. If enzyme-bound NAD^+ existed in equilibrium with a finite amount of the free coenzyme, the latter might act as

Table 1: Estimation of the NAD^+ Content in the Extract Prepared by Incubating Recombinant Sarcosine Oxidase at 37 °C for 30 min

	NAD^+ concentration (μM)	
sample	alcohol dehydrogenase assay[a]	absorbance at 260 nm[b]
enzyme extract	7	9
	21	21
	31	31
NAD^+ standard	3	4
	11	12
	22	24
	49	50

[a] The alcohol dehydrogenase assay was conducted under conditions as described in the legend to Figure 3. [NAD^+] was estimated on the basis of the increase in absorbance at 340 nm due to its conversion to NADH. [b] [NAD^+] was estimated on the basis of its absorbance at 260 nm (ϵ_{260} = 17.6 mM^{-1} cm^{-1}). The [NAD^+] in the enzyme extract was estimated after correction for the contribution due to FAD ($\epsilon_{260}/\epsilon_{450}$ = 3.27).

Table 2: Stoichiometry of NAD^+ in Recombinant Sarcosine Oxidase and Natural Enzyme from *Corynebacterium* sp. P-1[a]

	[FAD] (noncovalent)/[NAD^+]	
preparation	spectrophotometric analysis	HPLC analysis
recombinant enzyme	0.79	1.0
natural enzyme	1.1	1.1

[a] Enzyme extracts were prepared by denaturation with 3 M guanidine hydrochloride. In the spectrophotometric analysis, NAD^+ was estimated on the basis of its absorbance at 260 nm (see legend to Table 1), and FAD was estimated on the basis of its absorbance at 450 nm (ϵ_{450} = 11.3 mM^{-1} cm^{-1}). For the HPLC analysis, the ratio [FAD]/[NAD^+] was estimated from peak areas after correction for differences in the extinction coefficients of the chromophores at 260 nm (ϵ_{NAD^+} = 17.6 mM^{-1} cm^{-1}; ϵ_{FAD} = 37.0 mM^{-1} cm^{-1}). The extracts were chromatographed as in Figure 1.

a substrate for NAD^+-dependent enzymes. However, no hydrolysis of NAD^+ was observed after an overnight incubation of the enzyme with excess NADase under conditions which yielded complete hydrolysis of a control sample of free NAD^+. Similarly, no evidence for NAD^+ reduction was obtained after incubation of the intact enzyme with alcohol dehydrogenase plus ethanol for 16 h at 25 °C or after an additional 1 h at 30 °C. The alcohol dehydrogenase was still active at the end of this prolonged incubation since rapid NAD^+ reduction was observed when the temperature of the sample was raised to 37 °C.

Nicotinamides readily form reversible covalent adducts with cyanide. However, no reaction with NAD^+ bound to sarcosine oxidase was observed upon incubation of the enzyme for 80 min at 25 °C with 0.1 M cyanide at pH 8.5, as judged by the absence of an absorbance increase around 340 nm. Under similar conditions, the reaction of cyanide with free NAD^+ reached equilibrium (85% complex formation) within 35 min. The observed extent of complex formation agrees with a value estimated (88%) on the basis of the reported value for K_{eq} (200 M^{-1}, 15 °C) (Burgner & Ray, 1984).

Function of NAD^+ in Sarcosine Oxidase. In previous anaerobic titration studies at pH 8.0, reduction of the enzyme with substrate was found to consume 1 mol of sarcosine/mol of flavin reduced (Ali et al., 1991), suggesting that NAD^+ is not involved as a redox catalyst in sarcosine oxidation. The possibility that NAD^+ might be reduced in the presence of excess sarcosine was investigated in an experiment where the enzyme (8 μM) was reduced with 5 mM sarcosine in anaerobic buffer (10 mM potassium phosphate, pH 8.0, at 25 °C) and then denatured with 3 M guanidine hydrochloride while still under anaerobic conditions. The anaerobic denaturation step was designed to prevent any NADH reoxidation by electron transfer to oxygen via the flavin centers. The sample was made aerobic, microfiltered to remove protein, and subjected to HPLC analysis under conditions where NAD^+ (14.8 min) is readily separated from NADH (16.1 min). The enzyme extract contained NAD^+ but no NADH. The results show that NAD^+ in sarcosine oxidase is not reduced by substrate and is not in redox equilibrium with the flavins.

Sarcosine oxidase might be a bifunctional enzyme, catalyzing a second, unknown reaction involving NAD^+ as redox catalyst. As a preliminary test of this hypothesis, the products of sarcosine oxidation were screened to determine whether they might cause NAD^+ reduction when mixed with the enzyme under anaerobic conditions. No spectral changes were observed upon incubation with glycine, 5,10-CH_2-H_4-folate, or formaldehyde. No reaction was observed in trials with various other folate derivatives, including 5-CH_3-H_4-folate, 10-CHO-H_4folate, and 5-CHO-H_4folate.

DISCUSSION

In this paper we demonstrate that recombinant sarcosine oxidase and the natural enzyme isolated from *Corynebacterium* sp. P-1 contain a stoichiometric amount of tightly bound NAD^+ (1 mol of NAD^+/mol of protein). The NAD^+ is not lost during enzyme purification. It is not susceptible toward hydrolysis by NADase, reduction by alcohol dehydrogenase, or nucleophilic attack by cyanide. The failure to form a covalent adduct with cyanide suggests that the NAD^+ may be buried and inaccessible to solvent and/or that its reactivity with cyanide is suppressed by the protein environment. In studies with various free nicotinamides, cyanide complex stability was found to decrease as the redox potential of the analog became more negative and in more polar solvents (Hemmerich et al., 1977; Burgner & Ray, 1984).

The NAD^+ in sarcosine oxidase is not reduced by sarcosine nor is it in redox equilibrium with the flavins. Since NAD^+ could not be removed without denaturing the enzyme, we cannot exclude a possible catalytic and/or structural role of NAD^+ in sarcosine oxidation. Site-directed mutagenesis studies may prove useful in preparing NAD^+-free enzyme. Sarcosine oxidase contains two dinucleotide binding motifs located near the NH_2 termini of the α and β subunits (Chlumsky et al., 1995) which may participate in binding NAD^+ and the noncovalent FAD. The enzyme's covalent flavin is attached to a histidyl residue in the β subunit. It was identified in earlier studies as (8α-N^3-histidyl)-FAD (Kvalnes-Krick & Jorns, 1986), but recent studies show that the covalent flavin is present at the FMN rather than the FAD level.[2]

We considered the possibility that sarcosine oxidase might catalyze a second, unknown reaction involving NAD^+ as a

[2] A. Willie, D. E. Edmondson, and M. S. Jorns, unpublished observations.

redox catalyst but failed to obtain evidence for this hypothesis in a preliminary screen of compounds which included the products of sarcosine oxidation and various folate derivatives. The observed homology of the NH_2-terminal half of the α subunit with various opine oxidoreductases (Chlumsky et al., 1995) suggested that NAD^+ might participate in a reaction involving opine oxidation. However, NAD^+ reduction was not detected upon anaerobic incubation of the enzyme with various opines, including octopine, nopaline, and mannopine.[3]

In addition to sarcosine oxidase, other flavoenzymes have recently been found to contain adenine nucleotides. Mammalian and bacterial electron-transferring flavoproteins (ETF) contain 1 mol each of FAD and AMP (DuPlessis et al., 1994; Sato et al., 1993). ETF reconstituted from AMP-free apoenzyme exhibited the same activity as native enzyme, but the rate of reconstitution was accelerated in the presence of AMP. The presence of an additional coenzyme in trimethylamine dehydrogenase was discovered during analysis of the electron density map which revealed that the enzyme contained 1 mol of ADP in addition to its previously known prosthetic groups, covalently bound 6-*S*-cysteinyl-FMN and a [4Fe-4S] cluster. The ADP occupies a binding site analogous to the ADP moiety of FAD in glutathione reductase. Its function in trimethylamine dehydrogenase is unknown (Lim et al., 1988). Recent studies suggest that chicken liver L-2-hydroxy acid oxidase, an enzyme containing noncovalently bound FMN, may also contain NAD since treatment with NADase abolished activity of the enzyme with an alternate substrate, L-2-hydroxy-4-methylthiobutanoic acid (Ferjancicbiagini et al., 1995).

[3] L. J. Chlumsky, A. Willie, and M. S. Jorns, unpublished observations.

REFERENCES

Ali, S. N., Zeller, H. D., Calisto, M. K., & Jorns, M. S. (1991) *Biochemistry 30*, 10980−10986.

Bernofsky, C. (1980) *Methods Enzymol. 66*, 112−119.

Beutler, H. O., & Supp, M. (1983) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bermeyer, J., & Grassl, M., Eds.) pp 328−393, Verlag Chemie, Weinheim.

Burgner, J. W., & Ray, W. J. (1984) *Biochemistry 23*, 3620−3626.

Chlumsky, L. J., Zhang, L. L., Ramsey, A. J., & Jorns, M. S. (1993) *Biochemistry 32*, 11132−11142.

Chlumsky, L. J., Zhang, L., & Jorns, M. S. (1995) *J. Biol. Chem. 270*, 18252−18259.

DuPlessis, E. R., Rohlfs, R. J., Hille, R., & Thorpe, C. (1994) *Biochem. Mol. Biol. Int. 32*, 195−199.

Ferjancicbiagini, A., Dupuis, L., Decaro, J., & Puigserver, A. (1995) *Biochimie 77*, 249−255.

Hemmerich, P., Massey, V., & Fenner, H. (1977) *FEBS Lett. 84*, 5−21.

Jorns, M. S. (1985) *Biochemistry 24*, 3189−3194.

Jorns, M. S., & Hersh, L. B. (1975) *J. Biol. Chem. 250*, 3620−3628.

Klingenberg, M. (1985) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Bergmeyer, J., & Grassl, M., Eds.) pp 251−271, VCH, Weinheim.

Kvalnes-Krick, K., & Jorns, M. S. (1986) *Biochemistry 25*, 6061−6069.

Kvalnes-Krick, K., & Jorns, M. S. (1987) *Biochemistry 26*, 7391−7395.

Lim, L. W., Mathews, F. S., & Steenkamp, D. J. (1988) *J. Biol. Chem. 263*, 3075−3078.

Ljungdahl, L. G., O'Brien, W. E., Moore, M. R., & Liu, M.-T. (1980) *Methods Enzymol. 66*, 599−609.

Lowry, O. H., Passonneau, J. V., & Rock, M. K. (1961) *J. Biol. Chem. 236*, 2756−2759.

Rabinowitz, J. C. (1963) *Methods Enzymol. 6*, 814−815.

Sato, K., Nishina, Y., & Shiga, K. (1993) *J. Biochem. (Tokyo) 114*, 215−222.

Zeller, H.-D., Hille, R., & Jorns, M. S. (1989) *Biochemistry 28*, 5145−5154.

BI952374O