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A thermostable NADH oxidase from anaerobic extreme thermophiles

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A high-abundance NADH-oxidizing enzyme (NADH:acceptor oxidoreductase, EC 1.6.99.3) has been identified and isolated from a range of anaerobic extreme thermophiles, including strains of *Clostridium thermohydrosulfuricum* and *Thermoanaerobium brockii*. By use of a pseudo-affinity salt-promoted adsorbent, a nearly pure sample was obtained in one step; remaining impurities were separated by ion-exchange. The fully active purified enzyme contains FAD (two molecules per subunit of 75–78 kDa) and iron–sulphur, and is hexameric in its most active form. The reaction with oxygen is a one- or two-electron transfer to produce superoxide radical and H_2O_2; other acceptors include tetrazolium salts, dichlorophenol-indophenol, menadione and ferricyanide. The role of the enzyme is not clear; it was found not to be NAD:ferredoxin oxidoreductase, which is a major NADH-utilizing enzyme in these organisms.

INTRODUCTION

Obligate anaerobes have no obvious use for oxygen-consuming enzymes as part of their metabolism or energy generation; nevertheless there are reports of NADH and NADPH oxidases and associated peroxidase activities from anaerobes such as *Clostridium* and *Streptococcus* sp. (Dolin, 1959; Koike *et al.*, 1985; Schmidt *et al.*, 1986). In aerobic micro-organisms, NAD(P)H oxidase/dehydrogenase activities are generally associated with membrane fractions, and represent a side reaction of enzymes responsible for passing electrons from NAD(P)H through quinones and cytochromes to oxygen.

When testing extracts of strains of *Thermoanaerobium brockii*, *Clostridium thermohydrosulfuricum* and other less characterized extreme anaerobic thermophiles isolated from New Zealand hot springs (Patel *et al.*, 1986), we were initially unable to estimate accurately the activities of enzymes for which the assay involved donation of hydrogens by NADH (e.g. lactate dehydrogenase, pyruvate kinase), because of a rapid loss of NADH without any other substrate addition. This could be overcome by carrying out the procedures anaerobically, and was found to be due to a highly active NADH oxidase present in the extracts.

The present paper describes the isolation and characterization of this NADH oxidase from several strains of the thermophiles. The enzyme preparations isolated contain bound FAD and iron–sulphur, and reduce oxygen to superoxide and H_2O_2, but will reduce other acceptors even in the presence of oxygen. The enzyme thus resembles the NADH oxidases from *Bacillus megaterium* (Saeki *et al.*, 1985) and *Thermus aquaticus* (Cocco *et al.*, 1988). Experiments were designed to test the hypothesis that the true role of this enzyme might be as a ferredoxin:NAD^+ oxidoreductase (EC 1.18.1.3), which functions to transfer reducing equivalents [which originate either from the enzyme pyruvate:ferredoxin oxidoreductase (CoA-acetylating) (EC 1.2.7.1) or from pyruvate formate-lyase (EC 2.3.1.54)], from ferredoxin to NAD^+. The NADH oxidase enzymes from these anaerobes are thermostable, losing less than 50% of activity in 30 min at temperatures up to 80 °C. They are stable in air, and can be used to generate superoxide radical and H_2O_2 from NADH.

MATERIALS AND METHODS

Strains of organism

Samples of *C. thermohydrosulfuricum* Rt8.B1, *T. brockii* Rt8.G4 and Tok6.B1 and a strain known as TP10.B1 were generously provided by Professor H. W. Morgan, Waikato University, New Zealand. Samples of *Fervidobacterium nodosum* (Patel *et al.*, 1985) and an unidentified strain of a similar anaerobe from an Australian artesian bore, AB 10, were kindly provided by Dr. B. Patel, Griffith University (Nathan, Qld., Australia). They were cultivated in the medium described by Zeikus *et al.* (1979) at 68–70 °C, initially in 10 ml bottles, and then in 500 ml Schott bottles which were used as inoculum for a 10-litre fermenter (Braun Biostat E). Sugar substrates were glucose (0.5–3%) or fructose (0.5–2%). After fermentation was complete (16–20 h), the cells were harvested without maintaining anaerobic conditions. Yield varied between 90 and 120 g wet wt. from 10 litres.

Preparation of cell extracts

Extraction of the cells was by chemical lysis. To each 1 g of cell paste, 5 ml of 20 mM-potassium phosphate, pH 7.0, containing 0.2 mg of lyoszyme/ml, 5 μg of DNAase I/ml, 0.1% Nonidet P-40 and 10 mM-β-mercaptoethanol was added, and the mixture stirred at ambient temperature for 30 min. The lysate was centrifuged (20000 *g* for 20 min) to provide a clear deep-brown extract. All buffers used in the purification procedure contained 10 mM-β-mercaptoethanol.

Adsorbents

Epi-thiamin adsorbent was prepared by activating Sepharose CL4B (Pharmacia) with epichlorhydrin, and coupling with thiamin in a carbonate buffer, pH 10, by using 25 mg of neutralized thiamin hydrochloride to 1 g wet wt. of activated gel (Scopes & Porath, 1990).

Dye-ligand adsorbents were prepared as described by Atkinson *et al.* (1981).

Enzyme assays

NADH oxidase activity was routinely measured in an air-

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saturated 20 mM-potassium phosphate buffer at 25 °C containing 0.15 mM-NADH. Extract sample was added, and the initial rate of loss of A_{340} was noted. Activities are expressed in units of μmol of NADH oxidized/min at 25 °C unless otherwise indicated.

Evidence for the production of superoxide was obtained by using the ability of the enzyme to reduce cytochrome *c* in oxygen in the presence and absence of superoxide dismutase (McCord & Fridovich, 1969).

Activity as oxygen consumption was measured with an oxygen electrode (Rank Bros., Cambridge, U.K.).

Ferredoxin:NAD^+ oxidoreductase was measured as described by Blusson *et al.* (1980) and Jungermann *et al.* (1973).

Other techniques

H_2O_2 formation was measured as described by Thurman *et al.* (1972).

Flavin content was determined after deproteinization with trichloracetic acid, by using an absorption coefficient of 11300 $M^{-1} \cdot cm^{-1}$ at 450 nm, and t.l.c. was used to identify the flavin components (Schmidt *et al.*, 1986).

Iron was measured by the method of Doeg & Zeigler (1962), using phenanthroline on acid-treated samples of the enzymes.

Gel electrophoresis was carried out in a Pharmacia Phast system. Staining for activity of NADH oxidase used 20 mM-potassium phosphate buffer, pH 7.0, with 2 mM-NADH and 2 mg of Nitro Blue Tetrazolium/ml, and similarly for NADPH oxidase.

Amino acid analysis was carried out on samples hydrolysed in 6 M-HCl in an evacuated tube for 22 h at 110 °C. No corrections were made for losses of serine or threonine, nor for incomplete hydrolysis of valine, leucine and isoleucine.

RESULTS

Purification of NADH oxidase

A large range of dye ligands was screened to find a selective adsorbent for the enzyme (Scopes, 1986). Screening was carried out at both 20 °C and 50 °C. In neither case was there a clear separation of NADH oxidase activity from other proteins on any dye column, despite the expectation that dyes would be most selective for an NADH-binding enzyme. Consequently an alternative screening was carried out using salt-promoted multifunctional adsorbents (Scopes & Porath, 1990). After adding 0.5 M-Na_2SO_4 to the extract, some 20 adsorbents were screened; several of these bound the enzyme totally. An adsorbent made by coupling thiamin to epichlohydrin-activated Sepharose was found to bind all the NADH oxidase activity, but less than 5% of the total protein in the extract. This was selected as the most suitable adsorbent for purifying the enzyme; the activity could be eluted with buffer lacking Na_2SO_4. Remaining impurities were removed by anion-exchange chromatography.

Cell paste (110 g) was extracted with 550 ml of buffer as described under 'Preparation of cell extracts', and Na_2SO_4 added to 0.6 M. The solution was passed through a 100 ml epithiamin column (8 $cm^2 \times 12$ cm) pre-equilibrated in 30 mM-potassium phosphate/0.6 M-Na_2SO_4 buffer, pH 7.0, at 200 ml/h. The column was washed with 200 ml of 25 mM-Tris buffer, pH 8, containing 0.5 M-Na_2SO_4. NADH oxidase was then eluted with Tris buffer lacking Na_2SO_4. The eluted activity was concentrated by ultrafiltration, desalted on Sephadex G-25 into the Tris buffer, and run on a DEAE-Trisacryl column (5 $cm^2 \times 5$ cm). NADH oxidase activity was eluted by a NaCl gradient at about 100 mM-NaCl (Fig. 1). The preparation was > 95% pure as judged by SDS/PAGE at this stage.

A summary of a purification from *T. brockii* cells is given in Table 1. The enzyme was also purified from the other strains of extreme anaerobic thermophiles; the behaviour on purification and the properties of the purified enzymes were all very similar.

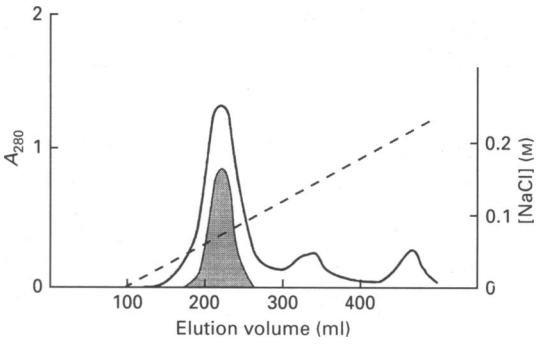

Fig. 1. Elution of NADH oxidase (*T. brockii*) from DEAE-Trisacryl column

——, A_{280}; – – – –, NaCl gradient. The position of the eluted enzyme is indicated by the shaded area; the peak of activity was 32 units/ml.

Table 1. Purification of NADH oxidase from 110 g wet wt. of *T. brockii* RT8.G4 cells

The specific activity of freshly prepared and purified enzyme from each source was in the range 23–28 units/mg.

	Protein, (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)
Extract	5700	2130	0.37	100
Eluted from thiamin column	120	1880	15.7	88
Eluted from DEAE-Trisacryl	50	1160	23	54

Molecular properties of NADH oxidase

Gel filtration of three freshly prepared samples on Superose 6 indicated a native molecular mass between 450 and 500 kDa. On PAGE (native gel) a single band (> 95%) was obtained, and in the presence of SDS the enzyme also gave a single band of estimated molecular mass 75–78 kDa (> 10 preparations). This indicates that the enzyme is probably hexameric in structure. The purified enzyme was brownish-yellow, indicating flavin content: the spectrum showed the characteristic double peaks at 380 and 450 nm of flavin, with some underlying additional adsorption. Various preparations from *C. thermohydrosulfuricum* and *T. brockii* were analysed for flavin after deproteinization. Samples with specific activities greater than 20 units/mg had a flavin content close to 2 mol/ml of subunit, but less active preparations (aged, or from frozen-stored cells) had a proportionally lower flavin content, as shown in Fig. 2. T.l.c. of acid-dissociated flavin showed that FAD was present, but no FMN. Iron analysis was carried out on several samples, giving values between 1.5 and 1.9 mol/mol of subunit. H_2S evolution on acidification suggested that the iron may be in the form of Fe_2S_2 clusters.

The amino acid analysis of *T. brockii* NADH oxidase is shown in Table 2, in comparison with that of the similar enzymes from *B. megaterium* and *Thermus aquaticus*. It is clear that these analyses are of similar proteins, but, in view of the 40%-larger subunit of the *T. brockii* enzyme, detailed comparisons are not justified. Attempts at *N*-terminal amino acid sequencing of several samples of our purified enzyme were not successful, indicating a blocked *N*-terminal amino acid.

Stored preparations lost some activity over time, and, on investigation by native focusing-gel electrophoresis, the band

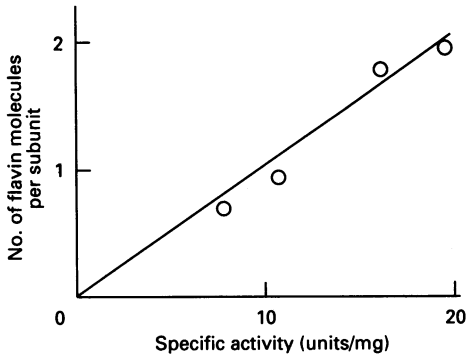

Fig. 2. Relationship between flavin content and specific activity of several preparations of *T. brockii* NADH oxidase

In all cases the enzyme was > 90% pure as judged by SDS/PAGE, but, owing to aging of stored cells and/or batch variation, some preparations when made had significantly lower specific activities than the maximum of 22–24 units/mg. The flavin content approached 2 molecules per subunit at maximum specific activity.

Table 2. Amino acid analysis of NADH oxidase from *T. brockii*, and comparison with the enzyme from other sources

Residue	Integral number per 78 kDa	*B. megaterium**	*T. aquaticus**
Asp	71	74	75
Thr	39	45	41
Ser	32	49	30
Glu	89	76	111
Pro	36	27	28
Gly	67	74	62
Ala	66	63	68
Cys	5	7	7
Val	57	50	58
Met	7	18	10
Ile	57	52	44
Leu	54	71	55
Tyr	24	14	18
Phe	22	24	22
His	13	15	14
Lys	46	41	31
Arg	34	23	35

* Converted to 78 kDa for comparison, from Saeki *et al.* (1985) and Cocco *et al.* (1988).

corresponding to the hexamer (450 kDa) decreased in amount, with an increase in a band at 250 kDa which appeared to be a trimer, and smaller amounts of possibly dimeric and monomeric components, all of which stained for activity with NADH/Nitro Blue Textrazolium. In addition, some samples showed an aggregated component close to 700 kDa which may be a nonamer. The hexamer and putative trimer can be separated by high-resolution ion-exchange chromatography or gel filtration, and the trimer had a specific activity that was approx. 60% of that of the hexamer.

Kinetic properties of NADH oxidase

By using a preparation from *C. thermohydrosulfuricum* Rt8.B1 in the standard assay mixture, saturated with air ($[O_2]$ = 0.22 mM), the K_m for NADH was determined to be 19 ± 3 μM at 25 °C (mean of six determinations). At 1 mM-NADH, with an oxygen electrode the K_m for oxygen was determined to be 0.21 mM. At 50 °C the K_m for NADH was 30 ± 5 μM (mean of four) and the apparent $V_{max.}$ was 2.1-fold higher. These rates cannot be compared meaningfully, as the oxygen was below saturation. By using the method of Thurman *et al.* (1972) on the product of the reaction it was shown that H_2O_2 was produced.

In anaerobic conditions, the enzyme would catalyse the reduction of a number of one- and two-electron acceptors, including ferricyanide, tetrazolium salts, menadione and dichlorophenol-indophenol. The K_m values for dichlorophenol-indophenol and *p*-iodonitrotetrazolium with the *C. thermohydrosulfuricum* and *T. brockii* enzymes were in the range 60–100 μM. In aerobic conditions these acceptors were still reduced, competing well with oxygen. In addition, cytochrome *c* was reduced. Since this could be the result of superoxide radicals reducing cytochrome *c*, the rate of reduction in air in the presence and absence of superoxide dismutase was compared (McCord & Fridovich, 1969). Cytochrome *c* reduction was abolished in the presence of superoxide dismutase, indicating that superoxide radicals are an immediate product of oxygen reduction. By comparing the rate of reduction of cytochrome *c* with the rate of oxidation of NADH, we estimate that the ratio of superoxide radical to H_2O_2 formed is approx. 1:2.

Experiments using luminol, with NADH/NADH oxidase in place of hypoxanthine/xanthine oxidase as described by Baret *et*

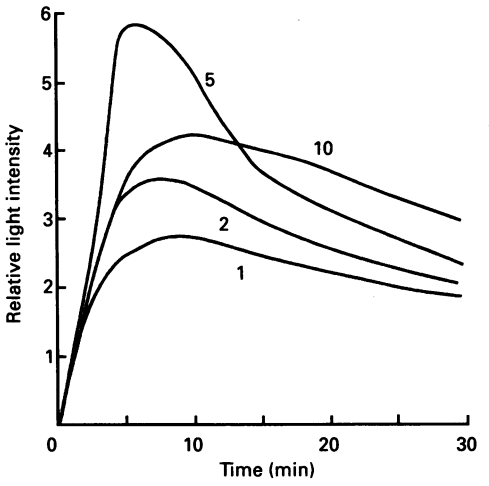

Fig. 3. Light production via the Fenton reaction and luminol, as described by Baret *et al.* (1990)

NADH was constantly regenerated by using ethanol and alcohol dehydrogenase in a pH 10.0 Na_2CO_3 buffer containing 5 μM-$FeCl_3$, 100 μM-EDTA, 20 μM-luminol and 0.1 unit of NADH oxidase/ml. Light was detected with a Hitachi model 204 fluorimeter without excitation. NADH level was varied as indicated, from 1 to 10 μM; peak light emission was at 5 μM. Addition of sodium perborate (20 μM) to generate H_2O_2 had no effect. Addition of horseradish peroxidase increased the light output up to 10-fold (result not shown).

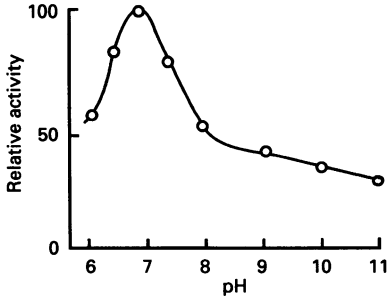

Fig. 4. pH/activity plot for NADH oxidase from *T. brockii*

Buffers used were: pH 6–7, 25 mM-K_2HPO_4 adjusted with acetic acid; pH 7.2–8, 25 mM-Tris/HCl; pH 9–11, 25 mM-glycine/HCl. NADH concentration was 0.15 mM.

al. (1990), resulted in light production, further confirming the superoxide formation. In this method, superoxide radicals and H_2O_2 undergo a Fenton-type reaction with complexed Fe^{3+} ions to form hydroxyl radicals, which in turn react with luminol to generate light (Fig. 3). We found that, in contrast with the situation using xanthine oxidase (Baret *et al.*, 1990), which exclusively forms superoxide radicals, it was not necessary to add peroxide to the system with NADH oxidase. Thus both superoxide and H_2O_2 must be products of the enzymic reaction.

The enzyme is specific for NADH, having no activity (< 1 %) on NADPH. The pH optimum for oxygen reduction is in the range 6.5–7.0, with considerable activity persisting up to pH 11 (Fig. 4).

Enzyme stability

The thermostability of the enzyme was tested in phosphate buffer, pH 7–8 (as measured at 25 °C). The 30 min half-life occurred at 80–85 °C, depending on strain, age of preparation and pH of incubation. Long-term stability in the cold was moderately good at pH values above 6. Activity declined to about 50 % of the original in a few weeks, owing to hexamer dissociation (see above), but then remained fairly constant for a long period, with a slow decline owing to proteolysis, as observed by SDS/PAGE. The survival of activity was not directly affected by air, although it was necessary to include β-mercaptoethanol in the buffers during purification to obtain a fully active enzyme.

Investigation of ferredoxin:NAD^+ oxidoreductase activity

In view of the large amount of activity of this enzyme present in the cells, its function must be important. One of the major enzymes present in these thermophiles that has not been isolated and described in detail is the ferredoxin:NAD^+ oxidoreductase, principally responsible for transferring reducing equivalents from reduced ferredoxin (obtained in oxidizing pyruvate) to NAD^+. The possibility that our enzyme might have this activity was investigated. Three tests were all negative; these included the assay as described by Jungermann *et al.* (1973), a system utilizing hydrogen and a hydrogenase in a crude extract of *Butyribacterium methylotrophicum* to reduce the ferredoxin, and a direct measurement of the enzyme by the method described by Blusson *et al.* (1980) in the crude extract before and after passing through the thiamin column while maintaining anaerobic conditions. Each of these tests employed acetyl-CoA as activator, as reported by Jungermann *et al.* (1973). In the third experiment, 100 % of the ferredoxin activity passed through the thiamin column, whereas 80 % of the oxidase activity was adsorbed and subsequently eluted.

DISCUSSION

Enzyme systems that catalyse the oxidation of NADH and NADPH with the formation of water are well known and widely distributed; electrons are transferred from NAD(P)H through various acceptors to oxygen. On resolution of these systems, a specific receptor for the reducing equivalents, e.g. ubiquinone or a flavoprotein, is usually identified as the direct substrate of the 'NADH dehydrogenase' or 'diaphorase' enzyme. However, in some cases a non-physiological direct oxidation of NAD(P)H without additional acceptors can occur to produce water (Schmidt *et al.*, 1986; Koike *et al.*, 1985) or occasionally H_2O_2 or superoxide radical (Saeki *et al.*, 1985). The best-known example of the latter is the physiologically significant NADPH oxidase of neutrophils, in which the superoxide radical is produced to destroy foreign material.

In anaerobes, it is unlikely that NADH oxidation by oxygen has a physiological role. Flavoproteins in which the flavin moiety is exposed to the solvent can readily transfer electrons to oxygen to form superoxide radical or H_2O_2; indeed, reduced riboflavin will do the same (Weintraub & Frankel, 1972). In aerobic organisms, the flavin portion of flavoproteins is usually protected by being buried, so that these unwanted and dangerous oxygen derivatives are not produced. But in anaerobes, it is not necessary for the flavin to be so protected, so on unnatural exposure to air, reduced flavin may be able to transfer electrons directly to oxygen. The NADH-utilizing enzyme described here does just that. The FAD molecules, which normally transfer electrons to some as yet unknown acceptor, are presumably exposed to the solvent. Consequently in an aerobic environment it rapidly carries out the NADH–oxygen reaction.

We have found NADPH oxidase activity in cell extracts of all of these thermophiles, at about 20 % of the level of NADH oxidase. But activity staining on a native electrophoresis gel showed that this is spread over many components, whereas the NADH activity is 80 % localized to the hexameric enzyme described here.

Although the role of the enzyme (other than the possibility that it could act as an oxygen scavenger) is unknown, one possible candidate was ferredoxin:NAD^+ oxidoreductase. The activity of this enzyme in crude extracts is comparable with the oxidase activity (Lamed & Zeikus, 1980). However, we have eliminated the possibility that our enzyme as prepared has any ferredoxin activity, using three different tests. Nevertheless it remained possible that exposure to air during isolation destroyed this activity, while retaining (or even creating) the oxidase activity. By carrying out the first stages of purification in anaerobic conditions we were able to separate the two activities. The ferredoxin enzyme was not in fact highly sensitive to air, but the assay must be carried out anaerobically.

The purification scheme for NADH oxidase, which is mainly a single-step process using a hydrophobic adsorbent, is highly selective for this enzyme. The structure of the thiamin adsorbent is presumably a linkage through the carbon atom of the thiazoline ring, since this is the nucleophile in normal thiamin reactions. There is no obvious reason why the structure of this adsorbent should 'fit' the NADH oxidase active site, and in fact, since attempts at elution with NADH have not been notably successful, it may be that the main binding is due to a chance interaction on another part of the enzyme's surface. The success of the method is a further illustration of the usefulness of screening a large range of randomly chosen adsorbents to find the optimum one for the purification being carried out.

Although most NADH dehydrogenases are membrane-bound, we do not suspect that the enzyme described here is an integral part of a membrane. But the hydrophobic character of NADH oxidase, as indicated by its behaviour on hydrophobic adsorbents, does suggest that it could interact loosely with the cell membrane. On the other hand, the amount of enzyme present, in some strains up to 3 % of the soluble protein, may be too much for all to be accommodated on the cell membrane. These thermophile cells are easily lysed by using lysozyme plus a small amount of non-ionic detergent which releases the intracellular contents, and most of the enzyme is solubilized in parallel with protein release.

The structure of NADH oxidase indicates a large molecule with probably six identical subunits. These subunits tend to fall apart to trimers and smaller fragments on storage, possibly owing to weakened hydrophobic interactions at ambient temperatures; these are less active than the hexameric form.

Although our enzyme has stability characteristics similar to the *Thermus aquaticus* enzyme (Cocco *et al.*, 1988), there are major differences. These include the fact that our enzymes are from anaerobes; they have two FAD molecules per subunit, not

one; the subunits are larger and form hexamers rather than dimers; and the reaction with oxygen produces superoxide radical, as well as H_2O_2. Moreover our enzyme is present at levels over 100-fold greater per g of cell protein, with the purified enzyme's specific activity some 30-fold higher.

The presence of both iron–sulphur and FAD in this enzyme allows single-electron transfer to create the superoxide radical, or to reduce single-electron acceptors such as ferricyanide. This NADH oxidase is particularly suited to study the detailed electrochemistry involved, as it can be isolated in large amounts, and is adequately stable both on long-term storage and at high temperatures. Potential applications of this enzyme in biosensors and in chemiluminescent diagnostic kits require investigation.

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