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Stabilisation of the NAD$^+$-reducing soluble [NiFe]-hydrogenase from *Ralstonia eutropha* H16 through modification with methoxy-poly(ethylene) glycol

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ABSTRACT

It has lately been demonstrated that the NAD$^+$-reducing soluble hydrogenase from *Ralstonia eutropha* H16 (SH) is a promising catalyst for the regeneration of NADH in biocatalysed asymmetric redox reactions. Such reactions often require the presence of water-miscible organic solvents and ionic liquids to enable efficient application to organic synthesis. In this study, we investigated the influence of frequently used solubilisers such as dimethyl sulphoxide [DMSO] and Tris (2-hydroxyethyl) methylammonium methylsulphate [MTEOA][$MeSO_4$] on the activity and stability of SH. The stability of the enzyme was significantly improved by covalent attachment of methoxy-poly(ethylene) glycol (mPEG). This modification led to significant increase of the half-life time from 0.1 to 0.5 h in the presence of 10% (v/v) isopropanol. Interestingly, no stabilisation was observed for ionic liquids, while the activity of SH increased by up to 45.5%. The mechanism(s) underlying these effects are discussed.

1. Introduction

The soluble [NiFe]-hydrogenase [SH; EC 1.12.1.2] of the strictly respiratory β-proteobacterium *Ralstonia eutropha* H16 is capable of catalysing the H_2-mediated reduction of the cofactor nicotinamide adenine dinucleotide (NAD$^+$) [1]. This reaction has optimal atom efficiency and only protons are formed as side-products. In contrast to most other [NiFe]-hydrogenases, the SH is catalytically active even under aerobic conditions [2]. Consequently, SH provides a promising alternative for the regeneration of NADH in industrially important biocatalysed reductions such as the synthesis of chiral hydroxy acids [3,4], amino acids [5,6] or alcohols [7–9]. In fact, compatibility of the SH with various alcohol dehydrogenases has already been shown [10–13]. Moreover, it has recently been demonstrated that the catalytic efficiency as well as the specific activity of the SH are clearly superior over the established regeneration catalyst formate dehydrogenase [FDH; EC 1.2.1.2] from *Candida boidinii* [13]. A major drawback for the technical application of SH, however, is its limited stability in synthetic batches. At optimum conditions for NAD$^+$ reduction (i.e. 35 °C, pH 8.0), a half-life of the purified SH of 5.3 h was observed, which decreased due to agitation as exerted by standard stirring devices and in the presence of various salts [13]. The reason for the limited stability is most likely related to the complex molecular structure of this heterohexameric metalloenzyme, which has a HoxHYUFI$_2$ subunit composition and harbours multiple cofactors including the Ni–Fe catalytic centre, one [2Fe–2S] cluster, at least three [4Fe–4S] clusters and two flavin mononucleotides (FMN) [14–19]. Detailed information on the quaternary structure, however, is not available yet, which excludes a rational design of SH composition towards an improved stability.

A common strategy for the protection of native enzymes from various detrimental effects is the chemical modification of the protein surface with polymers such as poly(ethylene) glycol [20–23]. For example, mPEG-modified benzaldehyde lyase showed improved stability towards the molecular and interfacial toxicity of various organic solvents, which was assigned to the amphiphilic character of methoxy-poly(ethylene) glycol [23].

In this study, the effects of a covalent modification of SH with mPEG to accessible surface residues were investigated with regard to the NAD$^+$-reducing activity and long-term stability. The experiments were preformed both in pure aqueous solution and in the presence of selected water-miscible organic solvents and ionic liquids, which are frequently used to improve the solubility of reactants in water [24–29]. Furthermore, a systematic study of the effects of these solubilisers on the NAD$^+$-reducing activity and long-term stability of native SH was performed.

Abbreviations: SH, soluble hydrogenase; IL, ionic liquid; mPEG, methoxy-poly(ethylene) glycol; DMF, dimethyl formamide; DMSO, dimethyl sulphoxide; [EMIM][$EtSO_4$], 1-ethyl-3-methylimidazolium ethylsulphate; [MTEOA][$MeSO_4$], Tris (2-hydroxyethyl) methylammonium methylsulphate.

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2. Experimental

2.1. Chemicals and enzyme

The NAD^+-reducing soluble hydrogenase (SH) with a $HoxHYUFI_2$ composition was overproduced in *Ralstonia eutropha* on the basis of an overexpression plasmid carrying the *hoxFUYWI-hypA2B2F2CDEXA* genes. The HoxF subunit was equipped with an N-terminal *Strep*-tagII, and the SH protein was purified by *Strep*-Tactin affinity chromatography using 50 mM K-PO_4, 5% glycerol pH 7.0 as the standard buffer [19]. The specific H_2-dependent NAD^+-reducing activity of the purified enzyme was around 30 units mg^{-1} protein. Tris(2-hydroxyethyl) methylammonium methylsulphate ([MTEOA][$MeSO_4$]) was a gift from ITMC at RWTH Aachen (Prof. W. Leitner). Page Blue™ and Page Ruler™ were purchased from Fermentas (St. Leon-Rot, Germany) and riboflavin 5-monophosphate sodium salt dihydrate (FMN) from Sigma (St. Louis, USA). All other chemicals were purchased from Carl Roth (Karlsruhe, Germany) and were of the highest available purity.

2.2. SH activity assay

H_2-driven reduction of NAD^+ to NADH was monitored spectrophotometrically at 340 nm (Cary 50, Varian) in a total volume of 1 mL of H_2-saturated (0.79 mmol L^{-1} at 35 °C) Tris/HCl buffer (50 mmol L^{-1}, pH 8.0) containing 1 mmol L^{-1} NAD^+ and 1 μM FMN. The reaction was started by addition of an appropriate amount of enzyme (typically 1 μL corresponding to an enzyme concentration of 26 nmol L^{-1}). The slope of the reaction was linear for at least 1 min. The molar extinction coefficient of NADH at 340 nm is 6220 L mol^{-1} cm^{-1} [30]. One unit of SH activity is defined as the amount of enzyme catalysing the reduction of 1 μmol NAD^+ per min. Results of activity measurements presented in this paper represent the mean of at least three independent experiments.

2.3. Determination of SH stability

The stability of SH was determined by measuring enzyme activity over time under aerobic conditions. If not stated otherwise, the stability assays were performed in 50 mmol L^{-1} Tris/HCl at pH 8.0 and 35 °C, except when the influences of pH and temperature, respectively, were determined. At appropriate time intervals, aliquots were withdrawn and the activity was assayed as described above. The enzyme stability is expressed by the half-life ($t_{1/2}$), i.e. the time after which the enzyme retains 50% of its initial activity under defined conditions, as calculated by Eq. (1),

$$t_{1/2} = \frac{\ln 2}{k} \quad (1)$$

where k is the rate constant of deactivation (h^{-1}) and was determined by linear fitting according to first-order rate law kinetics, which resembles the behaviour of SH [31]. Values for half-lives are given as the mean of at least three independent experiments.

2.4. Chemical modification of SH with methoxy-poly(ethylene) glycol (mPEG)

Activation of the $mPEG_{5000}$ derivative was performed according to [23,32] using *p*-nitrophenylchloroformate as activating agent. The final precipitate was dried under vacuum and stored at −20 °C. The activation degree of the purified mPEG derivative was 54.7%, which was determined spectrophotometrically by measuring *p*-nitrophenol (λ = 400 nm) released upon alkaline hydrolysis. *p*-nitrophenyl-$mPEG_{5000}$-carbonate ^1H NMR ($CDCl_3$, 200 MHz, 20 °C): δ (ppm) = 8.53–8.63 (m, 2 H), 8.28–8,35 (m, 2H), 3.69 (s, 4H), 3.37 (s, 3H).

Chemical modification of purified SH with mPEG was performed for 1 h at 4 °C in a Tris/HCl buffer (50 mmol L^{-1}, pH 8.0) with a 500-fold molar excess of activated $mPEG_{5000}$. During the chemical modification the solution was stirred at 400 rpm. For the removal of *p*-nitrophenol from protein samples, buffer exchange was performed using ultrafiltration devices equipped with a membrane (size exclusion: 10 kDa). Samples of mPEG-SH were applied to activity measurements and SDS-PAGE for characterisation.

2.5. SDS-PAGE of mPEG-SH

Denaturating polyacrylamide gel electrophoresis was performed according to Lämmli [33]. Separating gels had a concentration of 10% (w/v) polyacrylamide, stacking gels a concentration of 5% (w/v). Protein bands were stained with Coomassie blue dye.

3. Results and discussion

Based on the previously reported observations on SH sensitivity in the presence of shear forces and salt loads [13], a systematic study on the performance of SH in the presence of water-miscible solubilisers was performed in order to complete the available data on the influence of reaction conditions with particular importance for technical application.

As the organic solvents, DMF, DMSO and isopropanol, which were shown to have favourable effects on oxidoreductase catalysed reactions [34,35], were selected. In addition, two ionic liquids (ILs), [EMIM][$EtSO_4$] and [MTEOA][$MeSO_4$], were investigated considering the emerging potential of this type of solvent in the development of sustainable conversion processes [26–29]. Both ILs were deliberately chosen to contain sulphate because a stabilising effect of this ionic function on SH had been observed previously [13].

Organic solvents and ionic liquids were added to the aqueous enzyme solution to final concentrations of 10 and 25% (v/v) and 2.5, 5 and 10% (v/v), respectively, which represent typical ranges for solubilisers in enzyme-catalysed reactions [36–38]. Furthermore, optimum conditions for NAD^+ reduction by SH (35 °C, pH 8.0) [1,13] were chosen for the experiments. Both the data obtained in this study and in previous studies [13] were used to benchmark the effects of SH modification with mPEG.

3.1. Effects of organic solvents and ionic liquids on SH activity and stability

The solvents investigated in this study had generally diminished both the NAD^+-reducing activity and stability of SH (Table 1). At a solvent concentration of 25% (v/v), a residual activity (15.4% (±7.3)) was only detected in the presence of DMSO; whereas there was no activity at all at 25% (v/v) DMF and isopropanol. At a solvent concentration of 10% (v/v), a maximum residual activity of about 50% was obtained in the presence of DMSO; the most detrimental effect with only about 7% residual activity was exerted by DMF.

In full agreement with these findings, the half-life of SH at a solvent concentration of 10% (v/v) decreased from 5.3 h to about one hour, when DMSO was used as solvent, and to about six minutes in the presence of DMF or isopropanol, respectively (Table 1). In this respect it is important to mention that a similar sensitivity has been reported for many enzymes [e.g. 39–41].

In the presence of the chosen ILs, a similar impact was observed in terms of SH activity, which decreased with an increasing concentration of IL in water (Table 1). The highest residual activity of about 94% was obtained in the presence of 2.5% (v/v) [EMIM][$EtSO_4$]. However, upon increasing the concentration of this IL to 10%, a significant decrease of SH activity to a residual value of about 29%

Table 1
Relative activity and half-life of native SH in the presence of water-miscible organic solvents and ionic liquids [50 mmol L^{-1} Tris/HCl, pH 8.0, 35 °C].

Solvent content [% (v/v)]	Residual activity [%]	Half-life [h]
Without solubiliser		
0	100	5.3 (±0.7)
Organic solvent		
DMF		
10	6.7 (±1.4)	0.1 (±0.01)
25	0	n.d.
DMSO		
10	50.9 (±1.7)	1 (±0.01)
25	15.4 (±7.3)	n.d.
Isopropanol		
10	22.5 (±4.4)	0.1 (±0.01)
25	0	n.d.
Ionic liquid		
[EMIM][$EtSO_4$]		
2.5	94.3 (±0.4)	1 (±0.1)
5	67 (±2.2)	0.3 (±0.03)
10	29 (±0.4)	0.01 (±0.00)
[MTEOA][$MeSO_4$]		
2.5	73.2 (±3.9)	7.1 (±0.8)
5	67 (±1.6)	2.2 (±0.3)
10	61.8 (±2.9)	0.9 (±0.2)

was observed. In contrast, the initial loss of activity in the presence of small concentrations (2.5% v/v) of [MTEOA][$MeSO_4$] was more severe (about 73% residual activity), but the decrease of this activity upon further addition of the IL to a final concentration of 10% (v/v) less dramatic. In the latter case, a residual activity of about 62% was obtained.

Remarkably, the half-life of SH increased by a factor of 1.4 to about 7 h in the presence of 2.5% (v/v) [MTEOA][$MeSO_4$] in the reaction assay (Table 1). However, the half-life decreased again in the presence of higher concentrations of the IL. At 10% (v/v) IL in the reaction system, the half-life was approximately 1 h, which is in the same range as in the presence of 10% (v/v) DMSO. In contrast, [EMIM][$EtSO_4$] had a generally negative effect on SH stability resulting in a decrease of the half-life to about 1 h, 30 min and 36 s at concentrations of 2.5, 5 and 10% (v/v), respectively. Again, these findings are in agreement with literature data, where both the decrease of enzyme activity in the presence of ILs [26,42,43] and the increase in stability have been reported [44–46]. They also correlate with the previous findings regarding the impact of salt ions on SH performance [13], which report a general, albeit quantitatively different decrease of activity in the presence of all investigated salts, whereas a general trend for effects on stability could not be observed. Taken into account that ILs are pure liquid salts, this correlation seems to be reasonable.

3.2. Stabilisation of SH by modification with mPEG

For SH stabilisation, methoxy poly(ethylene) glycol with a molecular weight of 5000 Da ($mPEG_{5000}$) and a chain length of 116 was covalently attached to accessible residues, most likely lysine, on the surface of the native enzyme (Fig. 1). The number of mPEG-molecules per molecule of enzyme could not be determined from these measurements. It seems that the smaller subunits, HoxU, Y and I, are largely unmodified, whereas mPEG was successfully attached to the large subunits HoxF and H. This might indicate that the lysines of HoxU, Y and I are buried inside the protein complex and thus are protected from modification. The occurrence of multiple protein bands suggests that the number of attached mPEG molecules is not identical for all enzyme molecules.

The mPEG-modified SH (mPEG-SH) retained 91.0 (±13.4) % of wild-type NAD^+ reducing activity (35 °C, pH 8.0). The half-life of 5.9 h (± 0.1) is quite comparable to that of native enzyme (5.3 h (±0.7)) [13]. Thus, the modification hardly affects the SH performance at standard conditions.

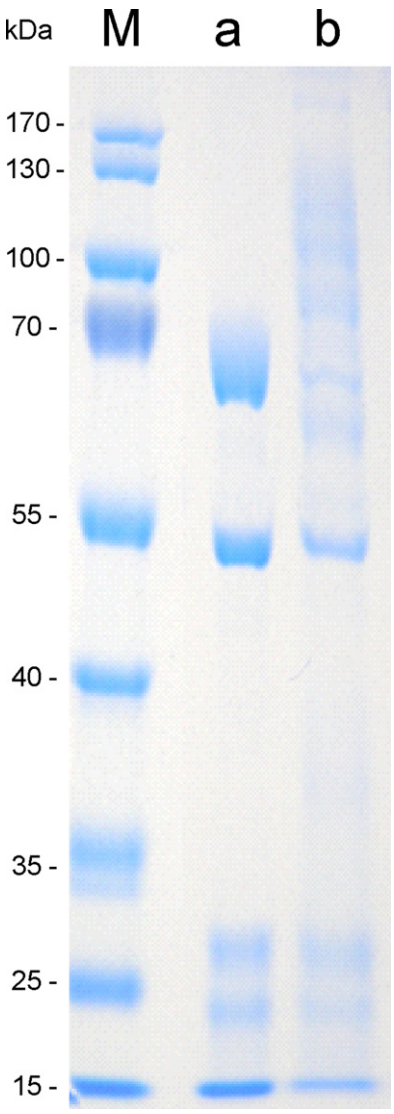

Fig. 1. Modification of SH with mPEG. SDS-PAGE of native (a) and mPEG- (b) SH. [M: marker, subunits of the native SH: HoxF 67 kDa, HoxH 55 kDa, HoxU 26 kDa, HoxY 23 kDa, HoxI 2 × 18.6 kDa]. Smearing between mPEGylated protein relates to the surface-active properties of mPEG.

In contrast, positive effects of mPEG-modification on the performance of SH during agitation and in the presence of water-miscible solvents were observed. In a vessel stirred with a magnetic bar at 400 rpm, mPEG-SH displayed a half-life of 3.2 h (±0.3) compared to 1.9 h (± 0.1) of native SH. The absolute value, though, strongly depends on the size of the bar and vessel as well as on the depth of the solution.

In the presence of the water-miscible organic solvents DMSO, DMF and isopropanol, the mPEG SH showed a remarkably higher activity than the native enzyme (Fig. 2A). This was particularly evident for DMF, which has the most detrimental effect on the native SH (Table 1). At a concentration of 10% (v/v) DMF, the residual activity of mPEG-SH was 22.9% (±3.3) compared to 6.7 (±1.4) % of the native enzyme. This is an enhancement by a factor of 3.4. In the presence of 10% (v/v) DMSO and isopropanol, the activity was still enhanced by a factor of 1.4 and 2.0, respectively.

In agreement with these findings, mPEG-SH revealed a clearly increased stability in the presence of 10% (v/v) of all three organic solvents (Fig. 2B). Maximum half-life was still found in the presence

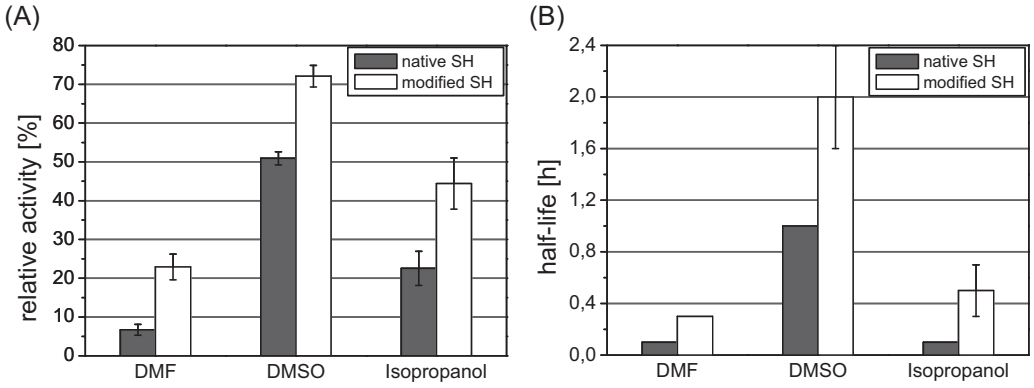

Fig. 2. Stabilisation effects of covalent attachment of mPEG on the SH in the presence of water-miscible organic solvents. Relative activity (A) and half-life (B) of native and mPEG-SH in the presence of 10% (v/v) water-miscible organic solvents [50 mmol L^{-1} Tris/HCl, pH 8.0, 35 °C; 100% activity corresponds to a specific activity of 30.2 units mg^{-1} (±1.0)].

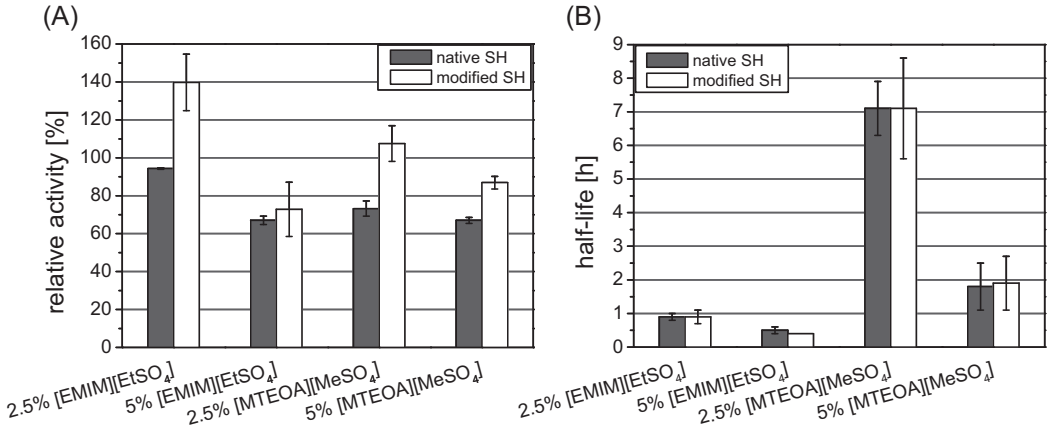

Fig. 3. Enhancement of relative activity by covalent attachment of mPEG on the SH in the presence of ionic liquids. Relative activity (A) and half-life (B) of native SH and mPEG-SH in the presence of 2.5 and 5% (v/v) water-miscible ionic liquid [50 mmol L^{-1} Tris/HCl, pH 8, 35 °C; 100% activity corresponds to a specific activity of 30.2 units mg^{-1} (±1.0)].

of DMSO (2.0 h (± 0.4)), which is by a factor of 2.0 higher than native SH. In the presence of DMF and isopropanol, the half-life was enhanced even by factors of 3.0 and 5.0, compared to half-lives of 0.3 h (±0.01) and 0.5 h (±0.02), respectively, of native SH under the same conditions.

The activity of mPEG-SH was again clearly superior over the native enzyme in the presence of the water-miscible ILs [EMIM][$EtSO_4$] and [MTEOA][$MeSO_4$] (Fig. 3A). In fact, at a concentration of 2.5% (v/v) IL, the activity of mPEG-SH was even higher than the activity of native SH in pure buffer. It was increased by 45.5% in the presence of [EMIM][$EtSO_4$] and by 34.3% in the presence of [MTEOA][$MeSO_4$]. At a concentration of 5% (v/v) IL, the residual activity of mPEG-SH was 72.8% (±14.3) compared to 67% (±2.2) of native SH and 86.9% (±3.3) compared to 67% (± 1.6) in [EMIM][$EtSO_4$] and [MTEOA][$MeSO_4$], respectively. These findings are in good agreement with results from literature, in which increased activities of PEG-modified enzymes are described in the presence of various ILs [43].

Surprisingly, modification with mPEG had no significant effect on the stability of SH in the presence of the investigated ILs (Fig. 3B). This strongly indicates that the effects of these solvents on activity and stability are due to different mechanisms and corresponds to the occasionally observed controversial effects of dissolved salts on the activity and stability of SH [13].

The stabilising effect of mPEG is usually assigned to an increased degree of hydration on the surface of the enzyme due to the hydrophilic properties of PEG, along with a reduction of enzyme flexibility due to the interaction of the hydrophobic parts of PEG with hydrophobic parts on the protein surface [23,47–51]. This would result in the formation of a rigid cage around the enzyme, decreasing the susceptibility towards forces inducing defolding. The size and strength of this cage would depend on the number of PEG molecules attached per enzyme molecule. It can be envisioned that PEG provides successful protection as long as it can keep the cage intact, which in particular requires maintaining its hydration degree. The findings in this study indicate that the protection capability of PEG is sufficient up to a certain concentration of polar molecules, such as water-miscible organic solvents. In the presence of molecules with a high water binding strength, however, PEG fails to maintain hydration even at low concentrations of these substances. Assumedly, this is what happens in the presence of ionic liquids.

4. Conclusion

The results in this study demonstrate that the SH is a promising NADH-regeneration system that is functional even in the presence of low to moderate concentrations of solubilisers such as DMSO and [MTEOA][$MeSO_4$]. However, the covalent attachment of mPEG improved activity and stability in organic solvents and the activity in ILs. Furthermore, the stability during agitation was considerably enhanced by covalent modification of the enzyme surface with mPEG. This is an important and promising result with regard to

the application of SH in technical synthesis, where co-solvents are often required as entrainers or to enhance substrate and product solubility [24,25].

Residues accessible for modification with mPEG seem to be located mainly on the large subunits of the hydrogenase and the diaphorase module, HoxF and HoxH, respectively. Trial of alternative or additional modifications might also be envisaged to take advantage from alternative stabilisation mechanisms, which might then also improve the stability in the presence of ILs. It has been reported in literature that SH might be stabilised by immobilisation on a solid support [10,52,53]. Additional or even synergistic stabilisation of SH might therefore also be achieved by appropriate immobilisation of covalently modified SH.

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