The Preparation of Stable Enzyme–Coenzyme Complexes with Endogenous Catalytic Activity

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*N*⁶-(6-Aminoethylcarbamoylmethyl)-NAD⁺ was coupled to lactate dehydrogenase by using glutaraldehyde to form an active complex. The stability of this complex could be considerably improved by reduction with KBH₄, although this treatment caused a partial decrease in specific activity. NAD⁺ was also coupled directly to the enzyme by this method. All of these complexes exhibited an intrinsic activity in the absence of exogenous NAD⁺.

The potential of immobilized enzymes as catalysts in industrial, analytical and medical applications has long been realized, but many of the more useful enzymes require expensive coenzymes in stoichiometric quantities. If these systems are to be economic, it is essential that the coenzyme is recycled *in situ*. The principal technique has been to attach the coenzyme covalently to a variety of soluble supports by the *N*-1, *N*-6 or *C*-8 position of the adenine ring to facilitate retention of the coenzyme within the appropriate reaction system [see Lowe (1978) for a review]. A similar approach has been to co-immobilize an enzyme and coenzyme on Sepharose 4B to give a catalytic complex with no requirement for exogenous coenzyme (Gestrelus *et al.*, 1975).

A particularly attractive scheme is to couple a coenzyme analogue directly to an enzyme with either glutaraldehyde (Venn *et al.*, 1977) or carbodi-imide (Mansson *et al.*, 1978). These methods are interesting from the practical aspect of conserving coenzyme and also from a mechanistic point of view as possible models of prosthetic-group enzymes. However, the method using coupling with glutaraldehyde produces only a partially stable derivative.

In the present paper the reported instability of glutaraldehyde coupling is investigated, and a method is described for the direct attachment of unmodified NAD⁺ to lactate dehydrogenase, based on previously published observations that glutaraldehyde linkages can be stabilized by borohydride reduction (Gacesa & Whish, 1977, 1978).

Materials and Methods

NAD⁺ and pig heart lactate dehydrogenase (EC 1.1.1.27) were obtained from Boehringer Corp.,

Abbreviations used: *N*⁶-NAD⁺ = *N*⁶-(6-aminoethylcarbamoylmethyl)-NAD⁺.

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Lewes, Sussex, U.K. Phenazine ethosulphate and 2,6-dichlorophenol-indophenol were obtained from Sigma, Poole, Dorset, U.K. Sephadex G-50 (fine grade) was purchased from Pharmacia, London W.5, U.K. Aqueous glutaraldehyde (25%, w/v) and all other chemicals were obtained from BDH Chemicals, Poole, Dorset, U.K., and were of AnalaR grade where available. *N*⁶-NAD⁺ was a generously given by Dr. Per-Olaf Larsson, Biokemi II, Kemicentrum, University of Lund, Sweden.

*N*⁶-NAD⁺ was coupled to lactate dehydrogenase and fractionated on Sephadex G-50 (fine grade) as previously described (Venn *et al.*, 1977). Alternatively it was coupled to the enzyme by this method and subsequently reduced with KBH₄ by adjusting the reaction mixture to pH 8.5 with 0.2M-NaOH and adding solid KBH₄ to 0.25M. After 15 min at room temperature (22°C) effervescence had ceased, and the yellow solution was applied to a column (1.5 cm × 55.0 cm) of Sephadex G-50 (fine grade) equilibrated with 25 mM-sodium borate buffer, pH 10.0. Fractions (1.9 ml) were collected at a flow rate of 1 ml/min, and the reduced complex was eluted as a single peak at the void volume well separated from unbound *N*⁶-NAD⁺.

NAD⁺ was coupled directly to lactate dehydrogenase by a modification of the method of Gacesa & Whish (1977). NAD⁺ (75 μmol) was dissolved in a mixture of 2 ml of 50 mM-sodium acetate buffer, pH 5.7, and 3 ml of aq. 25% (w/v) glutaraldehyde, and allowed to react for 1 h at room temperature (22°C). The NAD⁺ adduct was precipitated with 15 vol. of ethanol (4°C), and collected by centrifugation at –5°C. The pellet was redissolved in 2 ml of 0.1M-sodium phosphate buffer, pH 7.5, added to 1.8 ml of lactate dehydrogenase solution (1.8 mg/ml) and coupling allowed to proceed for 15 h at 4°C. The solution was reduced with KBH₄ and chromatographed free of unbound NAD⁺ as described above.

An estimate of the protein and nucleotide contents of the various products was obtained spectrophotometrically.

metrically and enzyme activity was measured by using phenazine ethosulphate and 2,6-dichlorophenol-indophenol in a recycling assay system (Venn *et al.*, 1977). The intrinsic specific activity is defined as the activity expressed by the complex in the absence of added NAD^+ in units of A_{610}/min per mg of protein, whereas the specific activity is measured in the presence of 2mM-NAD^+ and expressed in the same units.

Results and Discussion

The results in Table 1 show that coenzyme was bound to lactate dehydrogenase by all three methods. Enzyme modified by the attachment of $N^6\text{-NAD}^+$ had a high molar ratio of cofactor/protein of 6.7 (non-reduced) or 6.6 (reduced), which corresponds to the maximum value obtained by Venn *et al.* (1977). However, the NAD^+ -enzyme complex had a ratio of only 3.5, despite a longer incubation time. No exact reason has been established for this, but the lack of a spacer arm on NAD^+ may mean that the primary amino group is less available for coupling because of steric hindrance by the adenine ring.

The specific activity of the non-reduced $N^6\text{-NAD}^+$ complex is lowered to 60% of the value obtained with the native enzyme (Table 1). A further decrease in specific activity also occurs after reduction with borohydride, but this disadvantage is outweighed by the improved stability of the complex. Although the specific intrinsic activity is only a small fraction of the specific activity of these preparations, it should be noted that this is primarily a reflection of the available concentration of nucleotide. Evidence for this is provided by assaying the native enzyme with only a 6-fold molar concentration of coenzyme. A specific activity of 6 A_{610} units/min per mg is obtained in comparison with a value of 500 A_{610} units/min per mg when the native enzyme is assayed in the presence

of 2mM-NAD^+ . Therefore the results obtained for the specific intrinsic activities of the complexes are of the same order of magnitude as the activity of the native enzyme when a similar concentration of coenzyme is available.

Little difference was observed between the specific activities of the reduced $N^6\text{-NAD}^+$ and reduced NAD^+ derivatives. This suggests that the mode of

Fig. 1. Stability of the enzyme-coenzyme complexes on storage

Non-reduced $N^6\text{-NAD}^+$ (○), reduced $N^6\text{-NAD}^+$ (△) and reduced NAD^+ (□) complexes were stored at 4°C in 0.1M -sodium phosphate buffer, $\text{pH} 7.5$, and the ratio of specific intrinsic activity to specific activity (α) was determined at various times. The complexes were rechromatographed on Sephadex G-50 to remove any nucleotide released during storage and the values of (α) redetermined (filled-in symbols).

Table 1. Properties of the lactate dehydrogenase complexes

The protein and nucleotide concentrations were estimated by u.v. absorbance at 290 and 266nm. Enzyme activities were measured in the absence (specific intrinsic activity) and presence of 2mM-NAD^+ (specific activity). With the native enzyme (specific activity 500 A_{610} units/min per mg) an apparent intrinsic activity was obtained (6 A_{610} units/min per mg) by incubating the enzyme with a 6-fold molar ratio of NAD^+ .

	Complex of lactate dehydrogenase with:		
	$N^6\text{-NAD}^+$ (non-reduced)	$N^6\text{-NAD}^+$ (reduced)	NAD^+ (reduced)
Protein concn. (μM)	3.83	4.40	3.60
Coenzyme concn. (μM)	25.8	29.0	12.7
Molar ratio of coenzyme/protein	6.7	6.6	3.5
Specific activity (A_{610} units/min per mg)	290.0	142.0	122.0
Specific intrinsic activity (A_{610} units/min per mg)	1.76	1.18	0.72
$10^3 \times$ Ratio of specific intrinsic activity to specific activity (α)	6.1	8.3	5.9

binding is similar in both cases despite the lack of a six-carbon spacer in the NAD^+ derivative. This is substantiated by the observation that the specific intrinsic activities for the reduced complexes are very similar when corrected for the amount of coenzyme bound in each case. Thus, if the specific intrinsic activity is divided by the molar ratio of cofactor/protein, then values of 0.18 ($N^6\text{-NAD}^+$) and 0.20 (NAD^+) are obtained. A slightly higher value of 0.26 was obtained for the non-reduced $N^6\text{-NAD}^+$ complex.

The results (Table 1) show that bound NAD^+ is slightly less effective as a coenzyme for lactate dehydrogenase than an equivalent concentration of free NAD^+ . Therefore it would be expected that any release of coenzyme from the complex would cause an increase in specific intrinsic activity of the preparation. The ratio (α) of specific intrinsic activity to specific activity is a useful way of expressing this, as an increase in this ratio (α) would indicate partial dissociation of coenzyme from the complex. Fig. 1 shows that when the various complexes are stored in 0.1M-sodium phosphate buffer, pH 7.5, at 4°C the non-reduced derivative shows a significant increase in the ratio (α), whereas the reduced complexes show very little change. The ratio (α) of the non-reduced derivative fell well below the original value after chromatography on Sephadex G-50, suggesting that some of the $N^6\text{-NAD}^+$ had dissociated from the complex. Evidence for this was provided by the elution profile (Fig. 2), where a peak co-chromatographing with $N^6\text{-NAD}^+$ was observed. The cofactor/protein ratio for the non-reduced $N^6\text{-NAD}^+$ complex also fell from 6.7 to 5.3 after chromatography, indicating a loss of 1.4mol of $N^6\text{-NAD}^+$ /mol of protein.

Different results were obtained with the reduced complexes. Neither the $N^6\text{-NAD}^+$ nor the NAD^+ derivative showed any marked change in the ratio (α) of enzyme activity over a period of 5 weeks, and only a small decrease in this ratio occurred after chromatography on Sephadex G-50 (Fig. 1). No free nucleotide could be detected in either of the reduced derivatives, and the resultant small change in the ratio (α) of enzyme activity was considered to be insignificant, as no measurable change in the molar ratio of cofactor to protein was observed.

The increased stability of the reduced complexes is a significant advantage and clearly shows that glutaraldehyde with subsequent borohydride reduction can be used satisfactorily as a reagent for coupling nucleotides directly to enzymes. The previous method of coupling NAD^+ to lactate dehydrogenase, which did not include borohydride reduction, resulted in a bond that was unstable over a period of weeks (Venn *et al.*, 1977). Previous work (Gacesa & Whish, 1977, 1978) has shown that nucleotides immobilized on to modified polysaccha-

Fig. 2. Rechromatography of the $N^6\text{-NAD}^+$ -lactate dehydrogenase complexes on Sephadex G-50

Samples (500 μl) of the stored complexes were chromatographed on columns (0.9 cm $\times$ 30.0 cm) of Sephadex G-50 (fine grade) equilibrated with 0.1M-sodium phosphate buffer, pH 7.5, and fractions (0.7 ml) collected at a flow rate of 0.3 ml/min. The non-reduced ($\circ$) and the reduced (Δ) complexes were measured by u.v. absorbance. Fractions 1-16 were measured at 280 nm, and fractions 17-31 were measured at 266 nm. The complexes had been stored for 30 days (non-reduced) and 35 days (reduced) before chromatography.

rides with glutaraldehyde can be stabilized by borohydride reduction. Evidence was provided to show that immobilization probably occurred by Schiff-base formation between glutaraldehyde and primary amino groups. The work reported in this paper has extended the technique to produce an integrated enzyme-coenzyme complex that is functional and appears to be as stable as the native components of the system. Furthermore the relatively complicated synthesis of $N^6\text{-NAD}^+$ (Lindberg *et al.*, 1973) can be avoided by the direct coupling of NAD^+ to the enzyme. Although a lower molar ratio of cofactor/protein was obtained, it should be possible to improve this by adjusting the reaction conditions.

The methods described in this paper provide an ideal model for studying the potential of enzyme-coenzyme complexes as integral catalytic units. Preliminary evidence indicates that the enzyme and coenzyme act intramolecularly and that the observed specific intrinsic activity is not an artefact caused by nucleotide release in the reduced complexes (Venn, 1977). This method should be applicable to a whole range of enzyme-coenzyme systems, provided that a suitable recycling system is available. There is also potential for using similar systems as models of prosthetic-group enzymes.

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