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Binding Studies of a Spin-Labelled Oxidized Coenzyme to Bovine-Liver Glutamate Dehydrogenase

Alt ZANTEMA, Wolfgang E. TROMMER, Herbert WENZEL, and George T. ROBILLARD

Department of Physical Chemistry, University of Groningen

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NAD^+ with a nitroxide piperidine ring linked to the NH_2 group of the adenine possesses full coenzymatic activity with glutamate dehydrogenase. Electron spin resonance spectra in the presence of glutamate dehydrogenase show mixtures of free and strongly immobilized spin-label. Binding studies in phosphate buffer demonstrate: (a) weak binary binding to the enzyme with a dissociation constant in the order of 2 mM; (b) an indication for negative cooperativity or different sites for binding to enzyme · 2-oxoglutarate, with dissociation constants in the order of 20–250 μM; (c) similar but much weaker binding to enzyme·2-oxoglutarate·ADP; (d) a strong positive cooperative binding to enzyme·2-oxoglutarate·GTP, dependent on the enzyme concentration.

Binding of phosphate to the enzyme with a K_d of about 20 mM or binding of pyrophosphate or tripolyphosphate with a K_d of about 2.5 mM enhances the binding of spin-labelled NAD^+ in the presence of 2-oxoglutarate. There is evidence that the binding sites for these phosphates coincide with phosphate binding subsites of GTP.

Many kinetic and regulatory properties of bovine liver glutamate dehydrogenase are known (for review see [1]). Based on binding and kinetic data a ligand exclusion model for the binding sites has been proposed [2], which could be confirmed or rejected if data concerning the distances between substrates, coenzymes and effectors were available. Such information can be obtained through studies of the influence of different paramagnetic probes on each other and on ligands followed by ESR and NMR spectroscopy [3]. In addition to covalently bound spin-labels [4] spin-labelled substrates, coenzymes and effectors may also be useful. In this case, however, it is essential to determine whether these analogues bind in a manner similar to the unmodified compound and possess similar biochemical properties. The spin-labelled analogue of oxidized coenzyme, shown in Fig. 1 has approximately the same K_m and V as NAD^+ with lactate dehydrogenase and in addition it acts like the unmodified coenzyme with alcohol dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase [5].

In the studies presented below the binding properties of this spin-labelled NAD^+ to glutamate dehydrogenase have been investigated and compared with those of NAD^+ which are quite difficult to obtain by conventional methods [6–8]. Special attention has been focused on the often observed differences in glutamate dehydrogenase behaviour in Tris *versus* phosphate buffers [9].

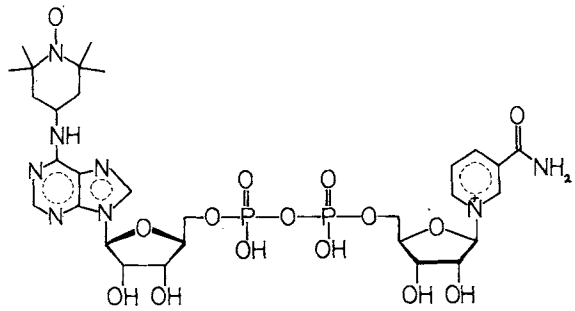

Fig. 1. *Formula of the spin-labelled NAD^+*

MATERIALS AND METHODS

Bovine liver glutamate dehydrogenase was obtained as a suspension in ammonium sulphate from Boehringer (Mannheim). The enzyme was dialysed at 4 °C first against a specified buffer in the presence of 0.05 M EDTA at pH 8.0, then several times against the buffer lacking EDTA. The cation was always the sodium ion. Enzyme concentrations were calculated

Abbreviations. ESR, electron spin resonance. In complexes the E stands for glutamate dehydrogenase and $slNAD^+$ for spin-labelled NAD^+.

Enzyme. Glutamate dehydrogenase or L-glutamate:NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3).

Fig. 2. *ESR spectrum of the spin-labelled NAD+ (A) free in solution, (B) bound to glutamate dehydrogenase.* Procedures described in the text

from the absorbance at 280 nm using an absorption coefficient of 0.93 cm^{-1} ml mg^{-1} [10]. The absorbance ratio at 280 and 260 nm was 1.91 ± 0.04. Molar contrations of the enzyme were always expressed as concentrations of protomers, with a molecular weight of 56000 [11]. Enzyme activity was measured by following the oxidation of NADH, 2-oxoglutarate and ammonia at pH 8.0 in 0.05 M Tris-HCl buffer containing 0.1 M NaCl and 0.1 mM EDTA at 25 °C. The absorbance at 340 nm was recorded on a 0–0.1 absorbance scale with a Zeiss PMQ II spectrophotometer equipped with a logarithmic converter. The concentrations in the assay were 9 mM 2-oxoglutarate, 140 mM NH_4Cl and 100 μM NADH. Under these conditions the specific activity was 65 ± 10 μmol min^{-1} mg^{-1}. The activity of the glutamate dehydrogenase was monitored after each binding study and was never significantly lower than at the beginning of the experiment.

Coenzymes, nucleotides, tripolyphosphate and 2-oxoglutarate (monosodium salt) were obtained from Sigma, glutarate and fumarate from Fluka and other chemicals from Merck, all of the highest purity available. Spin-labelled NAD^+ was prepared as reported [5]. Concentration of spin-labelled NAD^+ is determined by reduction with alcohol dehydrogenase [12].

All binding studies were performed at room temperature (22 ± 2 °C). ESR spectra were recorded on a Varian E-4 spectrometer. Binding studies were performed in a small (about 100 μl) quartz flat water cell (closed at the bottom). Small quantities (1 or 2 μl) of spin-labelled NAD^+ solutions were added with Drummond capillars to the enzyme solution in the cell and stirred with glass threads (use of metal threads gave a reversible reduction of the spin-label). Because the spin-labelled NAD^+ solution did not contain enzyme a dilution of the enzyme occurred (maxm 10%). In cases where the binding was enzyme-concentration-dependent, spin-labelled NAD^+ solutions containing enzyme were employed to prevent dilution.

Sedimentation velocity experiments were carried out at 20 °C with 59780 rev/min in a Beckman Spinco E analytical ultracentrifuge equipped with schlieren optics. The sedimentation coefficients were determined from photographs taken at various intervals.

ESR Spectra

The ESR spectrum of the spin-labelled NAD^+ free in solution is shown in Fig. 2A. In the presence of glutamate dehydrogenase and 2-oxoglutarate the spin-labelled NAD^+ has an ESR spectrum as shown in Fig. 3A. The spin-labelled NAD^+ in the presence of glutamate dehydrogenase, 2-oxoglutarate and GTP results in the spectrum shown in Fig. 3B. The two spectra in Fig. 3 clearly show the presence of the immobilized ESR spectrum of the bound spin-labelled NAD^+. The ESR spectrum of only the bound spin-labelled NAD^+, shown in Fig. 2B, was obtained by accumulating the spectrum of spin-labelled NAD^+ in the presence of glutamate dehydrogenase, 2-oxoglutarate and GTP, changing the sample for a free solution of spin-labelled NAD^+ and subtracting this free spin-labelled NAD^+ spectrum until no free signal remained. Some small residual peaks of the free signal are still observed. The distance between the outer peaks in Fig. 2B, $2T_{\parallel}$ = 65.5 ± 0.5 G, is the same for all complexes studied including the case of binding to E · 2-oxoglutarate · GTP where the viscosity was raised by a factor of 17 by means of glycerol. This indicates that the nitroxide has such a low mobility that changes in mobility are not reflected in the ESR spectrum.

Determination of Binding

Since the low-field peaks of both the bound and free spin-labelled NAD^+ are hardly influenced by one another, the peak heights of these signals have been used to calculate the ratio of the bound to free species. This procedure is allowable because a constant relationship exists between the peak height and area (determined by double integration [4]) for lines of a given shape. For the work described in this paper we find the above procedure more accurate than that of determining the fraction of bound label from the difference between the height of the free label peak and the measured peak hight of a label of known concentration. The objection to our method is that there may be bound spin-label molecules with a much

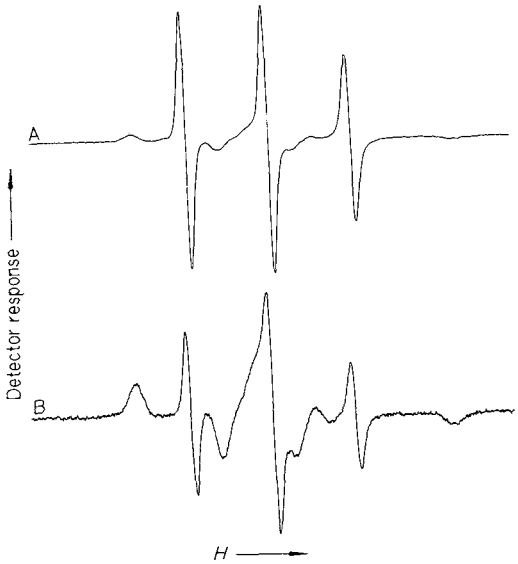

Fig. 3. *ESR spectra of spin-labelled NAD⁺*. (A) 180 μM spin-labelled NAD^+ in the presence of 11.4 mg/ml glutamate dehydrogenase and 70 mM 2-oxoglutarate; (B) 106 μM spin-labelled NAD^+ in the presence of 10.6 mg/ml enzyme, 70 mM 2-oxoglutarate and 1.5 mM GTP. In both spectra the enzyme protomers are half saturated with respect to spin-labelled NAD^+

higher mobility absorbing in the region of the free label peak. By using the method of free label peak height compared to the expected peak height we never found an indication for such bound spin-labelled NAD^+ molecules.

It is clear that determining the binding by means of the bound and free label peak heights, the largest errors occur in a very low saturation range, where the signal-to-noise ratio for the bound label is very low and in the high saturation range where the low-field peak of the bound label is in the flank of the free-label peak. For this latter reason it is also difficult to obtain accurate binding curves in the case of weak binding. The error bars given in the Scatchard plots represent the inaccuracy in the peak heights.

In all Scatchard plots having one kind of site or indicating negative cooperativity or different sites curves are drawn according to formula (1).

$$\frac{r}{S} = \frac{0.5}{K_1 + S} + \frac{0.5}{K_2 + S}. \qquad (1)$$

In this formula r is the fractional saturation, *i.e.* the bound spin-labelled NAD^+ ($= [slNAD^+]_b$) divided by the total enzyme concentration E, calculated with the molecular weight 56000, the molecular weight of the protomer. S is the free spin-labelled NAD^+ concentration ($= [slNAD^+]_f$) and K_1 and K_2 are two dissociation constants. An allowance for a possible lower saturation than a one-to-one binding is included in the given error limits of the dissociation constants.

RESULTS

To determine whether the spin-labelled NAD^+ can function as a normal coenzyme with glutamate dehydrogenase its activity was measured. At concentrations of 14 μM spin-labelled NAD^+ and 25 mM L-glutamate an activity of 84% was found relative to that obtained with the same concentration NAD^+. Thus along with other dehydrogenases studied [5] glutamate dehydrogenase is also capable of using this spin-labelled NAD^+ as a coenzyme.

When NAD^+ was added during an ESR spin-labelled NAD^+ binding study it appeared that there was a competition for the same sites with the NAD^+ binding being somewhat stronger.

BINARY COMPLEX

E · slNAD⁺

It is possible to observe the bound signal but the accuracy of determining the bound peak height is low and it is not possible to obtain a reasonable saturation of the enzyme. Assuming equal binding sites on all subunits the dissociation constant in 0.1 M phosphate pH 7.4 is in the order of 2 mM and in 0.05 M Tris-HCl, 0.1 M NaCl, 0.1 mM EDTA pH 7.4 in the order of 5 mM.

TERNARY COMPLEXES

E · 2-oxoglutarate · slNAD⁺

The binding of the spin-labelled NAD^+ to glutamate dehydrogenase changes drasticly in the presence of the product 2-oxoglutarate. The Scatchard plot for five different series of measurements in 0.1 M phosphate pH 7.4 are shown in Fig. 4. All series indicate a negative cooperativity or different sites with no systematic dependence on the glutamate dehydrogenase or 2-oxoglutarate concentration. The dissociation constants according to formula (1) are 21 ± 4 μM and 250 ± 100 μM. A Hill plot (not shown) gives $n_H = 0.75 \pm 0.07$.

A binding study to the same complex in 0.1 M phosphate and 0.1 M NaCl pH 7.4 gives the dissociation constants shown in Table 1. The extra NaCl decreases the binding of spin-labelled NAD^+. Studies starting in 0.1 M phosphate pH 7.4, with a spin-labelled NAD^+ saturation of the enzyme of 0.1 and adding concentrated NaCl solution give a decrease in $[slNAD^+]_b/(E \times [slNAD^+]_f)$, reflecting a decrease in binding. Even at 0.4 M NaCl no clear end point was obtained. The same experiment with NaAc does not give this decrease in binding, therefore chloride is responsible for it.

In addition to the effect of chloride there is a further weakening of binding in the presence of Tris instead

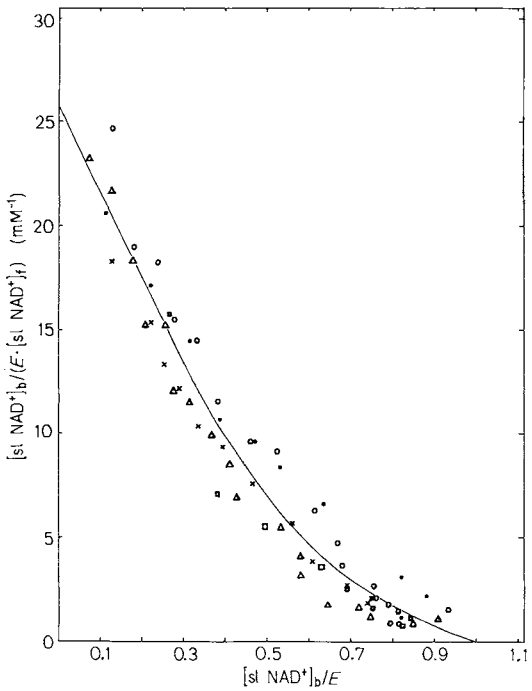

Fig. 4. *Scatchard plot of the binding of spin-labelled NAD$^+$ to glutamate dehydrogenase in the presence of 2-oxoglutarate in 0.1 M phosphate pH 7.4.* (○) 5 mg/ml enzyme, 100 mM 2-oxoglutarate; (△) 7 mg/ml enzyme, 80 mM 2-oxoglutarate; (●) 10 mg/ml enzyme, 70 mM 2-oxoglutarate; (□) 7 mg/ml enzyme, 80 mM 2-oxoglutarate; (×) 11 mg/ml enzyme, 70 mM 2-oxoglutarate. This last series is executed in the reverse way; starting with a high concentration spin-labelled NAD$^+$ and diluting with an enzyme · 2-oxoglutarate solution. The curve is calculated according to formula (1) with K_1 = 21 μM and K_2 = 250 μM

of phosphate. In comparing this difference between Tris and phosphate, we have circumvented the chloride effect by using a Tris buffer with monosodium 2-oxoglutarate as acid component. In this 0.1 M Tris/2-oxoglutarate buffer, pH 7.4, glutamate dehydrogenase was stable. The results of a binding study in this buffer are given in Fig. 5. Curves according to formula (1) indicate a K_1 = 47 ± 8 μM and a K_2 of 500–1700 μM.

The differences in strength of binding of spin-labelled NAD$^+$ to glutamate dehydrogenase in Tris *versus* phosphate buffer could arise from a specific binding of either of these substances. These possibilities were examined by monitoring the binding of spin-labelled NAD$^+$ to E · 2-oxoglutarate in phosphate buffer while titrating in Tris or in Tris buffer while titrating in phosphate (Fig. 6). The experiments are done at a saturation of about 0.1 spin-labelled NAD$^+$ per protomer glutamate dehydrogenase. The saturation of the enzyme does not change very much (less than 10%) during this kind of an experiment. Titration up to a concentration of 140 mM Tris/HAc or Tris/H_3PO_4 in a phosphate-buffered enzyme solution did not change the value of [slNAD$^+$]$_b$/(E × [slNAD$^+$]$_f$). Thus addition of Tris to the enzyme does not alter the binding of spin-labelled NAD$^+$ while Fig. 6 shows an enhancement in spin-labelled NAD$^+$ binding upon addition of phosphate. The binding of phosphate at different enzyme concentrations (see Fig. 6) does not show a significant enzyme concentration dependence. Assuming that phosphate binds to glutamate dehydrogenase and this binding has a simple relation to the binding constant of spin-labelled NAD$^+$ and therefore to the plotted

Table 1. *Dissociation constants for the binding of spin-labelled NAD$^+$ to E · 2-oxoglutarate in different buffers*
The dissociation constants were obtained from plots analysed to formula (1), making allowance for a possible slightly lower saturation than 1

Buffer	E	2-Oxo-glutarate	K_1	K_2
	mg/ml	mM	μM	
0.1 M phosphate pH 7.0	8.7	70	25 ± 4	320 ± 100
0.1 M phosphate pH 8.0[a]	5.3	95	21	90
0.1 M Tris/2-oxo-glutarate pH 7.0	12.6	100	55 ± 10	170 – 600
0.1 M Tris/2-oxo-glutarate pH 8.0[a]	11.4	65	52	150
0.1 M phosphate, 0.1 M NaCl pH 7.4	8.0	75	37 ± 7	260 ± 100
20 mM pyrophosphate/HAc pH 7.4	10.6	65	21 ± 4	200 ± 100

[a] No significant indication for different sites or negative cooperativity. Interpretation with one dissociation constant gives for phosphate 33 μM and for Tris/2-oxoglutarate 69 μM.

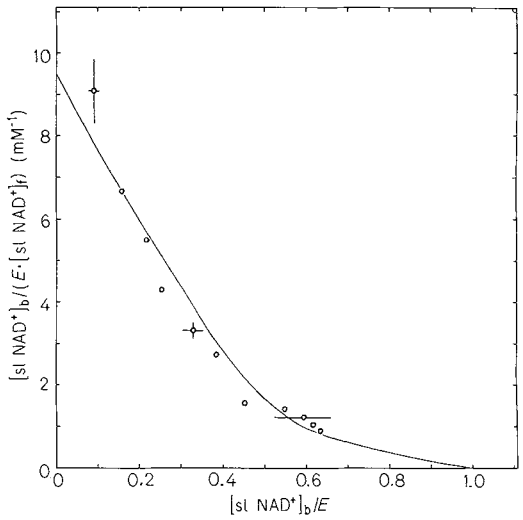

Fig. 5. *Sctachard plot of the binding of spin-labelled NAD$^+$ to glutamate dehydrogenase in 0.1 M Tris/2-oxoglutarate pH 7.4, 10.5 mg/ml enzyme. 95 mM 2-oxoglutarate.* The curve is calculated according to formula (1) with K_1 = 54 μM and K_2 = 1630 μM

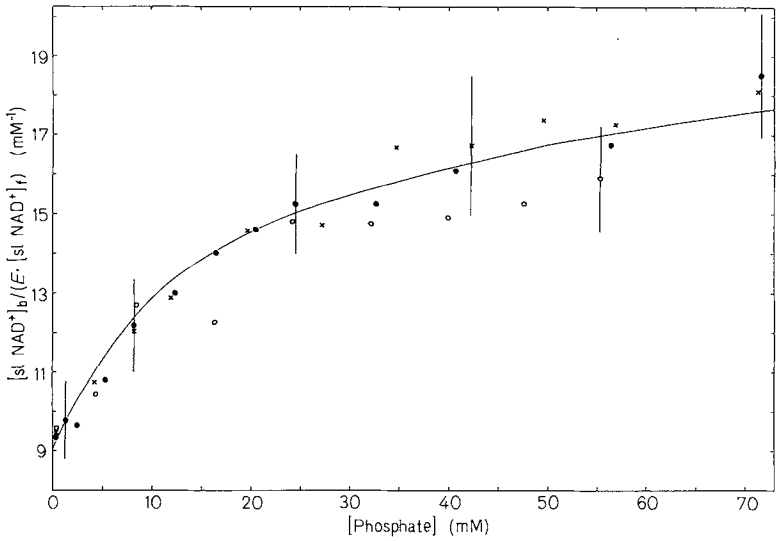

Fig. 6. *Influence of phosphate on the binding of spin-labelled NAD^+ to E · 2-oxoglutarate in 0.1 M Tris/2-oxoglutarate pH 7.4.* (×) 7.5 mg/ml enzyme, $[slNAD^+]_b/E = 0.12$; (●) 10.5 mg/ml enzyme, $[slNAD^+]_b/E = 0.11$; (○) 15.0 mg/ml enzyme, $[slNAD^+]_b/E = 0.11$. The curve is a binding curve with values of $[slNAD^+]_b/(E \times [slNAD^+]_f)$ from 9 to 19 mM^{-1} and $K_d = 16$ mM

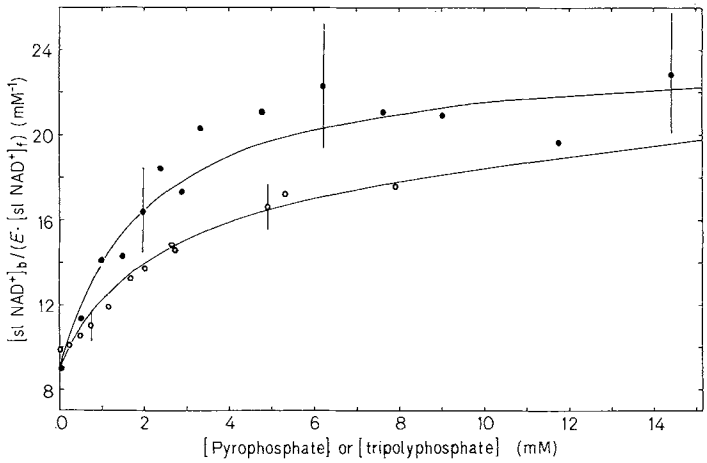

Fig. 7. *Influence of pyrophosphate (○), 15 ml/ml enzyme, $[slNAD^+]_b/E = 0.11$ and of tripolyphosphate (●), 16.5 mg/ml enzyme, $[slNAD^+]_b/E = 0.10$, on the binding of spin-labelled NAD^+ to E · 2-oxoglutarate in 0.1 M Tris/2-oxoglutarate pH 7.4.* The upper curve is a binding curve going from 9 to 24 mM^{-1} with $K_d = 2$ mM, the lower from 9 to 22 mM^{-1} with $K_d = 3$ mM

$[slNAD^+]_b/(E \times [slNAD^+]_f)$ we can determine the phosphate dissociation constant from the data in Fig. 6. The dissociation constant is 18 ± 8 mM. The large errors are caused by the fact that we do not know exactly the starting and end value along the ordinate. If instead of phosphate, pyrophosphate or tripolyphosphate is added the same effect is observed (see Fig. 7) only at a lower concentration. Pyrophosphate has an apparent dissociation constant of 3 ± 1 mM and tripolyphosphate of 2 ± 1 mM. To see whether this binding has an effect similar to phosphate, the binding of spin-labelled NAD^+ to E · 2-oxoglutarate was studied in a 20 mM pyrophosphate/HAc buffer pH 7.4. The dissociation constants according to formula (1) are shown in Table 1. As can be seen the result is similar to the binding observed in 0.1 M phosphate pH 7.4.

The binding of spin-labelled NAD^+ to E · 2-oxoglutarate in 0.1 M phosphate and 0.1 M Tris/2-oxoglutarate as well as the effect of phosphate have also been studied at pH 8.0 and pH 7.0. The dissociation constants are listed in Table 1. In each case the binding constants are only slightly changed. At pH 8.0 there is no significant indication for different sites or a negative cooperativity. Phosphate gives a similar effect as observed at pH 7.4 (Fig. 6). The phosphate dissociation constant is estimated to be in both cases 25 ± 10 mM.

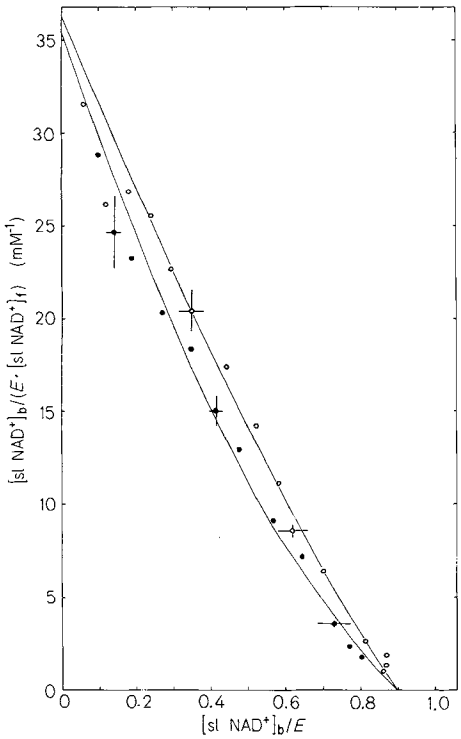

Fig. 8. *Scatchard plot of the binding of spin-labelled NAD$^+$ to E · glutarate in 0.1 M phosphate at (○) pH 7.4, 11.2 mg/ml enzyme, 95 mM glutarate or (●) pH 7.0, 10.9 mg/ml enzyme, 95 mM glutarate.* Curves according to formula (1) (with 0.45 instead of 0.5) with K_1 = 16.8 μM, K_2 = 46 μM (upper curve) and K_1 = 15.2 μM, K_2 = 76 μM (lower curve)

E · (substrate analogues) · slNAD$^+$

A study of the binding of spin-labelled NAD$^+$ to E · glutarate in 0.1 M phosphate pH 7.0 or pH 7.4 (Fig. 8) does not give a significant indication for a negative cooperativity. Straight lines with a saturation between 0.8 and 0.9 label per protomer give in both cases a dissociation constant of about 26 μM. This result certainly indicates a somewhat lower saturation than a one-to-one binding.

The binding of spin-labelled NAD$^+$ to glutamate dehydrogenase in the presence of 80 mM fumarate is similar to the binary binding of spin-labelled NAD$^+$ to glutamate dehydrogenase. This is in agreement with NMR results that fumarate binds well in the binary complex to the 2-oxoglutarate site but does not form an enhanced ternary complex (H. Koekoek, unpublished results).

QUATERNARY COMPLEXES

E · 2-oxoglutarate · slNAD$^+$ · activators

The binding of spin-labelled NAD$^+$ to E · 2-oxoglutarate is much weaker in the presence of ADP than in the absence of ADP. Both in 0.1 M phosphate pH 7.4 and in 0.1 M Tris/2-oxoglutarate pH 7.4 the

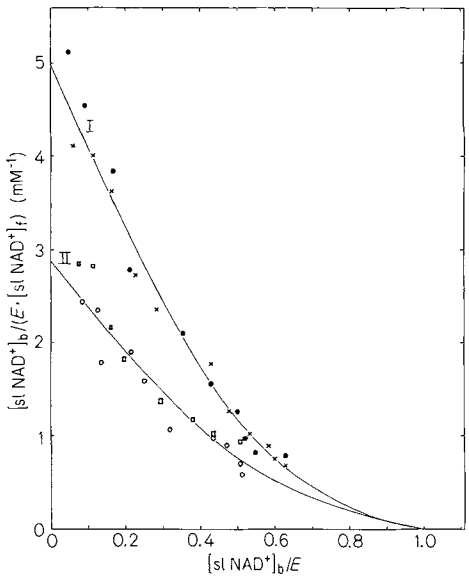

Fig. 9. *Scatchard plot of the binding of spin-labelled NAD$^+$ to E · 2-oxoglutarate · ADP.* (I) 0.1 M phosphate pH 7.4. (×) 11.0 mg/ml enzyme, 80 mM 2-oxoglutarate, 1.9 mM ADP; (●) 12.2 mg/ml enzyme, 65 mM 2-oxoglutarate, 0.9 mM ADP. The curve is drawn according to formula (1) with K_1 = 106 μM and K_2 = 1780 μM. (II) 0.1 M Tris/2-oxoglutarate pH 7.4, (□) 11.2 mg/ml enzyme, 90 mM 2-oxoglutarate, 1.9 mM ADP, (○) 10.9 mg/ml enzyme, 95 mM 2-oxoglutarate, 0.9 mM ADP. The curve is drawn according to formula (1) with K_1 = 189 μM and K_2 = 2370 μM

Table 2. *Dissociation constants for the binding of spin-labelled NAD$^+$ to different complexes of glutamate dehydrogenase*
The dissociation constants K_1 and K_2 were obtained from plots analysed according to formula (1), making allowance for a possible slightly lower saturation than 1. Both buffers were at pH 7.4

Complex	0.1 M phosphate		0.1 M Tris/2-oxoglutarate	
	K_1	K_2	K_1	K_2
	μM			
E · 2-oxoglutarate	21 ± 4	250 ± 100	47 ± 8	500 – 1700
E · 2-oxoglutarate · adenosine	35 ± 5	200 – 600	55 ± 10	350 – 1000
E · oxoglutarate · AMP	40 ± 5	220 – 730	80 ± 10	650 – 2000
E · 2-oxoglutarate · ADP	100 ± 10	600 – 1800	175 ± 15	880 – 2400

results (Fig. 9) show a slight indication for a negative cooperativity or different sites. The dissociation constants for these complexes, listed in Table 2, were again determined according to formula (1) and are, as mentioned, quite sensitive to the actual saturation. A similar but less dramatic effect has been detected with 2.8 mM AMP and a still smaller effect with 3.3 mM adenosine. In the cases of AMP and adenosine, however, the indication for different sites is clearer

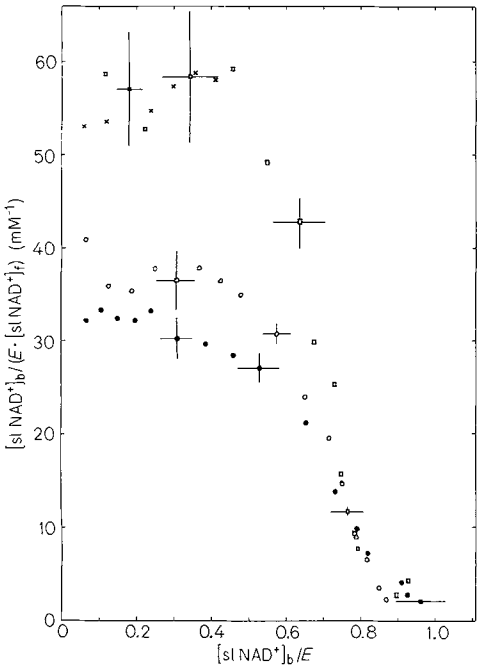

Fig. 10. *Scatchard plot of the binding of spin-labelled NAD⁺ to E · 2-oxoglutarate · GTP in 0.1 phosphate pH 7.4.* 0.8 mM GTP, 80 mM 2-oxoglutarate; (□, ×) 4.5 mg/ml enzyme, (○) 9.0 mg/ml enzyme, (●) 12.8 mg/ml enzyme

Table 3. *Sedimentation coefficients of glutamate dehydrogenase (6.2 mg/ml) in 0.1 M phosphate pH 7.4 at 20 °C in the presence of 2-oxoglutarate (80 mM) and different nucleotides*

Nucleotides added	*s*
	S
1 mM GTP	23.1
1 mM GTP + 1 mM NAD^+	12.3
1 mM GTP + 1 mM $slNAD^+$	11.8
4.5 mM GDP	23.0
4.5 mM GDP + 1 mM NAD^+	23.3 and 12.2

than in the case of ADP. The results are shown in Table 2.

E · 2-oxoglutarate · slNAD⁺ · inhibitors

The binding of spin-labelled NAD^+ to E · GTP is very weak with a binding constant in the same order as that observed for the binary binding to glutamate dehydrogenase. A dramatic difference has been found, however, when the binding of spin-labelled NAD^+ to E · 2-oxoglutarate · GTP was monitored. In Fig. 10 the binding in 0.1 M phosphate pH 7.4 is shown for three concentrations of glutamate dehydrogenase. It is clear that the Scatchard plot is strongly dependent on the glutamate dehydrogenase concentration. In all cases a positive cooperativity was found. The Hill coefficient is in these three cases 1.5 ± 0.15. The high saturation part of the Scatchard plot always indicates a dissociation constant of 6 ± 3 μM. The flat region at intermediate saturation in the Scatchard plot rises inversely with the enzyme concentration for concentrations from 3 to 12 mg/ml. The concentration of 0.8 mM GTP employed in the experiments in Fig. 10 may not be completely saturating, since a concentration of 1.5 mM gives a slightly stronger binding. Nevertheless the same effect is observed. In all Scatchard plots there is some indication at high saturation of a slight negative cooperativity; however, the accuracy is too low to get a significant determination of this effect.

The binding of spin-labelled NAD^+ to E · 2-oxoglutarate · GTP in 0.05 M Tris-HCl, 0.1 M NaCl and 0.1 mM EDTA pH 7.4 is the same as in 0.1 M phosphate pH 7.4. Therefore no effect of phosphate and chloride is observed. Using the substrate analogue glutarate instead of 2-oxoglutarate we found in 0.1 M phosphate pH 7.4 the same positive cooperativity.

Binding experiments of spin-labelled NAD^+ in 0.1 M phosphate pH 7.4 to E · 2-oxoglutarate and 2.6 mM GMP or to E · 2-oxoglutarate and 4.5 mM GDP give the same result as without the nucleotide.

SEDIMENTATION ANALYSIS

It is known that guanosine nucleotides can have a large effect on the association state of glutamate dehydrogenase. The results of a sedimentation analysis are shown in Table 3. We have used NAD^+ instead of spin-labelled NAD^+ assuming that the effects are similar. For the most pronounced effect we controlled this similarity. Clearly the complex E · 2-oxoglutarate · GTP is in the normal associated situation and the addition of NAD^+ as well as spin-labelled NAD^+ leads to a dissociation into hexamers. Using GDP instead of GTP the sedimentation in the presence of 2-oxoglutarate and NAD^+ shows two peaks about equally intense, indicating partial dissociation.

DISCUSSION

As is always the case, the reliability factor for information obtained from chemical modifications or from using analogues in place of true reactants and products is substantially higher when these procedures can be shown not to adversely disturb the macromolecule function or specific binding interactions. In the study presented here we found, for spin-labelled NAD^+, a good coenzymatic activity, an exclusion from binding by NAD^+ and, in the presence of 2-oxoglutarate and GTP, a dissociation of glutamate dehydrogenase into hexamers. Thus the bulky nitroxide substituent appears to generate no adverse steric hindrances upon binding to the active site of the enzyme. This is in agreement with studies on the 1:N^6-etheno derivative of NAD^+ [13]. The advantage

of using spin-labelled NAD$^+$ is that the binding with dissociation constants below 200 μM can be accurately measured over the whole saturation range. This is much more difficult using NAD(P)$^+$ [6,7].

A systematic error may be present in all binding data because of the accuracy in the determination of the area of bound and free spin-label peaks. As described, in all binding studies the same area relation has been used. If a systematic error is present it will be less than 20%. The saturation which is generally slightly lower than one site per protomer may be caused by this error.

Binding Studies

In studying the binding of spin-labelled NAD$^+$ in ternary or quaternary complexes we assume that the complex is formed before the spin-label binds. In general the concentrations employed have been sufficient to saturate all sites. We see no evidence for a compulsory order of binding. This is in agreement with equilibrium dialysis studies on similar complexes [6].

The results of our binding studies of spin-labelled NAD$^+$ in Tris and phosphate buffer are similar, except for the binding strengths. Because the binding in phosphate is stronger and therefore the results are more clear we will consider, in the following discussion, only the binding in 0.1 M phosphate.

There is no indication of more than one spin-labelled NAD$^+$ site per protomer. We do not see a binding to a second site [14]. Previous binding studies with NAD$^+$ [6,8] also gave no indication of binding to a second site; however a second molecule of NADH is observed to bind to the ADP site [1]. It should be mentioned that the 1:N^6-etheno derivative of NADH does not bind to the activator site, nor does that of ADP [13]. This is in agreement with the important role of the NH_2 on the adenine for binding ADP [18]. The absence of a free NH_2 in spin-labelled NAD$^+$ may be the reason for the lack of binding to the activating site.

Binary Binding

The binary binding of spin-labelled NAD$^+$ to glutamate dehydrogenase is too weak for accurate determinations since only a low saturation can be reached. From the results it is not clear what the actual saturation will be. The observed dissociation constant of 2 mM, assuming a one-to-one binding, is comparable with the reported values of 0.47 mM for NAD$^+$ at pH 7.0 [6], the calculated value of 2.2 mM for NAD$^+$ from spectroscopic measurements at pH 7.6 [7] and the calculated value from NMR measurements for NAD$^+$ of 1.1 mM at pH 7.4 in 0.05 M Tris, 0.1 M NaCl, 0.1 mM EDTA [4].

Ternary Complexes

It has been shown that the presence of 2-oxoglutarate strongly enhances the binding of NADP$^+$ [7]. While the same effect is found with spin-labelled NAD$^+$ we also observe a non-identity or a negative cooperativity of the binding sites. If this effect had been present in the study of Cross *et al.* [7] it would have been difficult to observe because of the very small optical change which occurs upon binding. Our results of course cannot distinguish between a negative cooperative effect or the fact that the sites are different. In our analysis we used the most simple case of different sites (formula 1) to give an impression of the changes in the dissociation constants.

It is possible that the observed negative cooperativity or different sites for the binding of spin-labelled NAD$^+$ to E · 2-oxoglutarate plays a role in the kinetics since it has been reported that the K_m of NAD$^+$ increases with increasing concentration [17]. However, binding data cannot determine kinetic pathways, which, in this case, seem to be governed by the off-rates from intermediate complexes [15, 16].

It is worthwhile to note that the spin-labelled NAD$^+$ binding in the ternary complex E · 2-oxoglutarate · slNAD$^+$ is comparable with the data from kinetics. The K_m values for NAD$^+$ are in the range from 4.1 to 220 μM, depending on the NAD$^+$ concentration.

Dalziel and Egan found a non-identity of sites or a negative cooperativity for the binding of NAD$^+$ to E · glutarate in 0.11 M phosphate pH 7.0 with dissociation constants in the order of 6 and 40 μM [6]. Our studies with spin-labelled NAD$^+$ in this complex (see Fig. 8) do not confirm this strong nonlinearity. The discrepancies will be discussed below.

Quaternary Complexes

The presence of activators adenosine, AMP or ADP with 2-oxoglutarate weaken the binding of spin-labelled NAD$^+$ to glutamate dehydrogenase. Nevertheless there is still evidence for negative interactions or different sites. The binding constants of the activators in these complexes are not known. However unless the binding of these activators is weaker than their binary binding [18] it is certainly reasonable to assume that AMP and ADP concentrations are saturating. The more pronounced effect towards ADP is in agreement with its larger activating effect [19]. It is remarkable that the Lineweaver-Burk plot of NAD$^+$ in the presence of ADP is linear [19,20] while we still see an indication for a negative cooperativity or different sites. It is possible, however, that a different step in the reaction mechanism has become rate-limiting.

The presence of the inhibitor GTP with 2-oxoglutarate causes a positive cooperative binding of spin-labelled NAD^+ to glutamate dehydrogenase. The sedimentation analysis (Table 3) shows that this positive cooperativity is accompanied by a dissociation into the free hexamers. Thus the spin-labelled NAD^+ binding to E · 2-oxoglutarate · GTP is stronger when the enzyme is dissociated into free hexamers. Because the association is dependent on the enzyme concentration [1] the binding curve must also be dependent on the enzyme concentration [21]. This is in accordance with our results (Fig. 10). A similar effect has been observed in the binding of NAD(P)H to E · GTP [22].

The binding of spin-labelled NAD^+ to E · glutarate · GTP gives the same very clear positive cooperativity. Dalziel and Egan found that NAD^+ binds to this complex at pH 7.0 with a negative cooperativity while the binding is accompanied by a dissociation into free hexamers [6].

Concerning the differences between our experiments and the experiments of Dalziel and Egan [6] we should mention two points. (a) If the reason for not observing the negative cooperativity upon binding of spin-labelled NAD^+ to E · glutarate is the bulky nitroxide group, the same results should be observed with 2-oxoglutarate; to postulate steric hindrance from the nitroxide in one case and not in the other would require a more complicated mechanism of conformational change upon binding (b) Dalziel and Egan observe a dissociation into free hexamers upon addition of NAD^+ to E · glutarate · GTP; for this binding we would expect a positive cooperativity as we found and as is found upon binding of NAD(P)H to E · GTP [22].

The binding of spin-labelled NAD^+ to E · 2-oxoglutarate · GDP does not show any effect of GDP. Even at the end of our binding study there is only a partial dissociation as shown by sedimentation (Table 3). During the binding there is no change from an associated to a complete dissociated situation and therefore we do not expect a positive cooperativity. GMP with 2-oxoglutarate also shows no effect on binding of spin-labelled NAD^+, however, we are not sure that the inhibitory site has been saturated.

Phosphate Binding

The study of the spin-labelled NAD^+ binding to glutamate dehydrogenase proved to be a sensitive probe in monitoring the effect of different buffers. As seen from Fig. 6 the phosphate ion has a large effect on the spin-labelled NAD^+ binding state to E · 2-oxoglutarate. This effect may be caused by a very weak binding or an aspecific ionic effect. The fact that chloride gives the reverse effect and acetate does not give any effect suggests a specific weak phosphate binding. This is strongly supported by the fact that pyrophosphate and tripolyphosphate give the same effect as phosphate but at a much lower concentration. The reported kinetic effects of phosphate [23–25] always suggest that the effect is an ionic one because other ionic substances give similar effects. It should be noted, however, that in general chloride ions, which we have shown to affect binding, were present in the other ionic substances. If this kind of effect happens in more complexes it is hard to predict what will be the effect on the kinetics. It certainly is possible that binding studies give contrary effects of phosphate and chloride, while the effect on the kinetics are similar. Recently, for an NADP-specific glutamate dehydrogenase from *Saccharomyces cerevisiae*, very marked phosphate effects have lead also to the postulation of a specific phosphate binding site distinct from the active coenzyme binding site [26].

The difference in binding of spin-labelled NAD^+ in phosphate and Tris/2-oxoglutarate buffer is still observed when, in addition to 2-oxoglutarate, an adenosine nucleotide is present. This suggests that the phosphate binding site does not coincide with one of the ADP phosphate sites. However the binding of spin-labelled NAD^+ to E · 2-oxoglutarate · GTP was found to be the same in 0.1 M phosphate and 0.05 M Tris-HCl, 0.1 M NaCl, 0.1 mM EDTA. No phosphate or chloride effect is observed, suggesting that the specific phosphate binding site coincides with one or more of the phosphate sites of GTP. This phosphate binding site corresponds with the model proposed by Goldin and Frieden [27]. The absence of a chloride effect in this case suggests that chloride might be effective by a competition for the phosphate site. However chloride titrations, as mentioned in Results, at different phosphate concentrations (0.05–0.25 M) hardly show any difference. Therefore the chloride effect appears to be an aspecific effect with respect to the phosphate effect.

With GMP or GDP plus 2-oxoglutarate the binding in 0.1 M phosphate is similar to the binding in the absence of the nucleotides. Some experiments in 0.1 M Tris/2-oxoglutarate in the presence of GMP indicate that the phosphate effect is present in this case, while in the presence of GDP it is not present. Especially for GMP, however, we are not sure that saturating concentrations have been achieved. These results are not clear enough to draw a conclusion about which phosphate subsite of GTP coincides with the phosphate site. An alternative possibility is of course that GTP introduces a conformational change which eliminates the phosphate binding site at a different part of the enzyme.

Our results strongly suggest that the stabilizing effect of phosphate for glutamate dehydrogenase [9]

is caused by a specific binding of phosphate. Further attempts to prove this binding and to locate the binding site are in progress.

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A. Zantema and G. T. Robillard, Fysisch Chemisch Laboratorium, Rijksuniversiteit te Groningen, Zernikelaan, NL-8002 Groningen, The Netherlands

W. E. Trommer, Institut für Organische Chemie, Biochemie und Isotopenforschung der Universität Stuttgart, Pfaffenwaldring 55, D-7000 Stuttgart 80, Federal Republic of Germany

H. Wenzel, Lehrstuhl für Biochemie, Abteilung für Chemie der Ruhr-Universität Bochum, Postfach 2148, D-4630 Bochum-Querenburg, Federal Republic of Germany