TRICINE RADICALS AS FORMED IN THE PRESENCE OF PEROXIDE PRODUCING ENZYMES

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1. Introduction

In view of findings [1] which showed a very narrow EPR spectrum, in tricine-dialysed spin-labeled pyruvate dehydrogenase complex compared to a normal spin-label spectrum, a more elaborate study was undertaken because the observed signals could not be explained unambiguously. As a possible explanation for the data, spin–spin interactions between the different spin-labeled species present, were suggested. It was considered that an organic radical could not be the cause of the signal observed because such radicals were believed to be either not formed or if formed, to be very unstable and thus not observable. However, recent experiments have shown the presence of a similar type of EPR spectrum, in the absence of the enzyme complex and spin label, but in a mixture of peroxide and tricine buffer at pH > 6. In this letter the originally observed signal is explained. Proof of the existence of a relatively stable nitroxide tricine radical is given. A theoretical description of the spectrum is presented.

2. Materials and methods

Pyruvate dehydrogenase complexes were isolated as described for *Azotobacter vinelandii* [2] and for *Escherichia coli* [3]. The *E. coli* enzyme was purified additionally on a hydroxyapatite chromatography column and was a gift from Mrs A. C. de Graaf-Hess. Glucose oxidase (grade III), catalase and superoxide-dismutase were obtained from Boehringer. Thiamine pyrophosphate (TPP), pyruvate, $MgCl_2$, K_2HPO_4, KH_2PO_4, tricine, ^2H_2O, NaO^2H and 30% peroxide were purchased from Merck. Dithiothreitol (DTT) was from Sigma, KO_2 from Alfa products.

Deuterated tricine was prepared by refluxing a 0.3 M solution in ^2H_2O with ~30 mM K_2HPO_4 at p^2H 13.0 for 5 days. From NMR (Perkin Elmer R-24B) it followed that after five days almost all 2-methylene protons were exchanged, while none of the other methylene protons were substituted.

EPR spectra were taken on a Varian E-3 spectrometer with a Varian Variable temperature Accessory V-4557. Samples in water were measured in quartz capillars (i.d. 0.8 mm, o.d. 3 mm).

Acetylation of the lipoyl moieties of the pyruvate dehydrogenase complex was performed by incubating the complex for 30 min at 0°C with Mg^{2+} (5 mM), TPP (0.5 mM) and pyruvate (5 mM). Oxidase activity was measured with a Clark-type electrode (Yellow Spring instruments); in addition to the acetylation mixture 0.1 mg/ml catalase was present.

Deacetylation of the enzyme complex was accomplished by adding EDTA (1–5 mM), CoASH (4–8 mM) and NAD^+ (4–8 mM). The reaction mixture was incubated for 30 min at 0°C, while in the anaerobic case also DTT was added (1–2 mM). After that time the reagents were removed either by dialysis against buffer or elution over a Sephadex G-10 column.

3. Results and discussion

3.1. *Radical formation under influence of peroxide*

If peroxide is added to tricine buffer at pH > 6, a time-dependent ESR signal (fig.1) is observed,

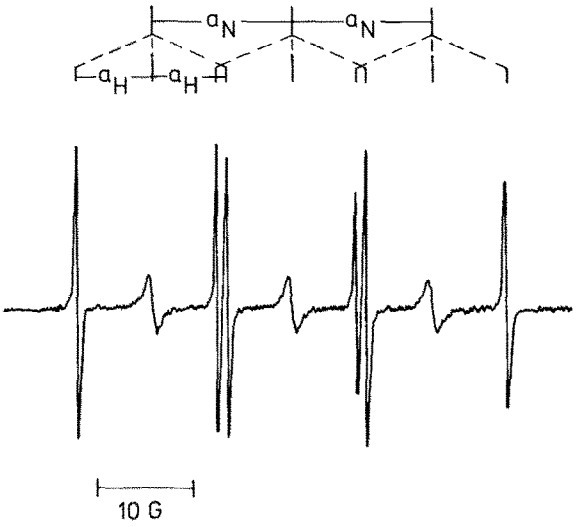


Fig.1. Tricine radical measured at room temperature. Tricine was 250 mM at pH 8.5, with 0.3% H_2O_2. Spectrum taken after 3.5 h at 4°C. a_N = 14.65 G, a_H = 7.85 G. EPR settings were: power 1 mW; modulation amplitude 0.02 G; gain = 2.5 × 10^5. The isotropic *g*-value was not calibrated, but is similar to the isotropic *g*-value of a nitroxide radical.

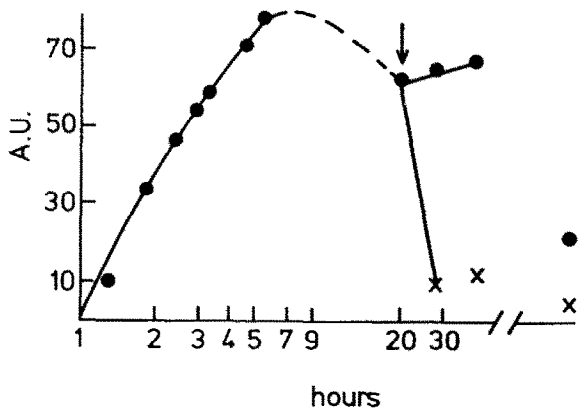


Fig.2. Time dependence of radical formation. Tricine was 300 mM at pH 7.0, with 0.3% H_2O_2. For convenience a logarithmic time scale was chosen. Time evolution at 4°C (• – •); the broken line is arbitrary. The *Y*-scale is in arbitrary units (A.U.). At the arrow either catalase (0.2 mg/ml) or superoxide dismutase (2 mg/ml) is added, or the sample is brought to room temperature. In all three cases a similar decrease, indicated by the (× – ×) is observed. After 4.5 weeks the rest signals were measured.

which also depends on tricine and peroxide concentrations, and pH. In fig.2 the dependence of the signal with time is given. Catalase or superoxide dismutase addition or elevation from 0–20°C at the indicated point leads to a decrease in time of the signal. The slight increase upon opening the vessel could be due to a new oxygen level in solution. The rest signal observed persists for several weeks. Clearly the radical formed is relatively stable. The pH optimum is ~pH 10, while below pH 6 no signal evolves, thus indicating the probable involvement of an N–H group.

The observation of the signal in spin-labeled pyruvate dehydrogenase complex [1] suggests the presence of pyruvate oxidase activity in the complex. Oxidase activity in Tris buffer present has been shown [2] in a partially-purified complex (before Biogel column) isolated from *A. vinelandii*. We tested whether this activity is present in tricine buffer with our purer preparation. Although a very low oxidase activity was observed the radical signal still evolved. The complex isolated from *E. coli*, corrected to the same FAD concentration, showed more oxidase activity and also a more intense radical signal evolved.

Flushing with oxygen, thus stimulating the oxidase activity, increased the signals. No signal was observed when the complex was incubated under identical conditions, but anaerobically in the presence of DTT.

Instead of peroxide being the reactant for radical formation, superoxide formed from peroxide under alkaline conditions could be the oxidizing agent. An experiment in which first a comparable amount of potassium superoxide and subsequently a much larger excess was added at pH 12.5 did not give any signal over 2 h. An identical solution of tricine to which peroxide was added developed in the same time as normal signal. To see whether final superoxide was involved in the complex via the flavin, since flavins are known to produce superoxide during oxidation reduction reactions [4], an experiment was done in which pyruvate dehydrogenase complex in tricine buffer was exposed to light for 20 min. No radical could be observed. Clearly peroxide is a more likely candidate for the oxidation of tricine. Furthermore the experiments suggest that the generation of the radical is linked to the oxidase activity of the pyruvate dehydrogenase complexes studied.

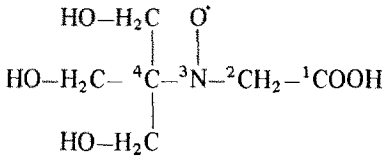
3.2. *Radical formation in labeled enzyme*

The signal in fig.1 is also observed when pyruvate dehydrogenase complex is spin-labeled in phosphate buffer and subsequently dialyzed against tricine buffer, as reported [1]. The signal observed of such a sample cannot be related directly to peroxide formed during oxidase activity because all oxidizing reagents were removed on the Sephadex G-10 column on which the unreacted spin-label was separated from the complex.

In this case deacetylation of the reacted sulfhydryl groups in the presence of oxygen and sulfhydryl reagent might give rise to the signal, since spontaneous oxygen-dependent deacetylation has been observed [5] in radioactive-labeling experiments. From fig.1 it seems that the reaction with peroxide is at least initially (pseudo) first order suggesting the formation of an intermediate which gives rise to the radical formation. Also the fact that superoxide dismutase leads to a decrease of the radical signal similar to catalase indicates that peroxide itself is not the sole reagent, and that an intermediate on the complex is involved. Both spin labeled and similar acetylated label-free complex do not show any signal after dialysis to tricine buffer if prepared under anaerobic conditions and deacetylated before exposure to air. Neither does a signal evolve when the complex, labeled or not, is acetylated anaerobically, followed by removal of the reactant on Sephadex G-10. Spin label alone in tricine does not give rise to the characteristic signal, even when purged with oxygen. These experiments illustrate that for the observed radical signal in pyruvate dehydrogenase complex, whether labeled or not, at least oxygen is necessary during the reduction with Mg, TPP and pyruvate and that neither pyruvate dehydrogenase complex alone, nor spin label alone gives rise to the signal in tricine.

3.3. *Nitroxide radical*

The origin of the signal remains to be answered. Besides the linewidth alternations in fig.1 the spectrum can be fitted in assuming 1 nitrogen hyperfine coupling of 14.65 G and 2 proton hyperfine couplings of 7.85 G as is illustrated in the stick spectrum of fig.1. This could be explained by assuming that the 3-NH of tricine is oxidized and forms an NO radical:



In order to check this, deuterated tricine was treated with peroxide identically and the resulting EPR spectrum is shown in fig.3. The quintets can be simulated with 2 ^{2}H hyperfine couplings of 1.2 G, while the nitrogen hyperfine coupling is still identical (rows 1, 2 of stick spectrum). The smaller peaks due to half-deuterated tricine, can be fitted with 1 H coupling of 7.85 G and 1 ^{2}H coupling of 1.2 G (rows 3, 4 in stick spectrum). Smaller contaminents are consistent with the double-protonated species. This all is clearly consistent with a simple deuterium replacement of the 2-methylene protons (7.85/6.51 G = 1.2 G).

The linewidth alternation in fig.1 can be explained by hindered rotation of this group [6]. The reason for this must be the acid group, most probably H-bridged to other tricine molecules. Indeed the linewidth of the broadened peaks at low pH (pH 0.8) decreases markedly. Figure 4 shows a high temperature spectrum in water at 90°C (lower spectrum) and a low temperature spectrum in 30% glycerol at –20°C (upper centrum). Variation of the viscosity shows a continuous change to the low temperature spectrum

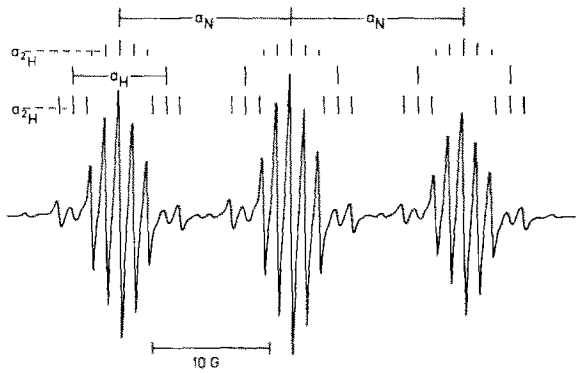


Fig.3. Deuterated tricine radical. Deuterated tricine was prepared as in section 2. To the reaction mixtures at pH 13 (tricine 300 mM), peroxide was added to final conc. 0.3%. The spectrum was taken after 2.5 h at 4°C. a_N = 14.65, $a_{^2H}$ = 1.2 G, a_H = 7.85 G. The EPR settings were: power 25 mW; modulation amplitude 0.032 G; gain 3.2 × 10^4.

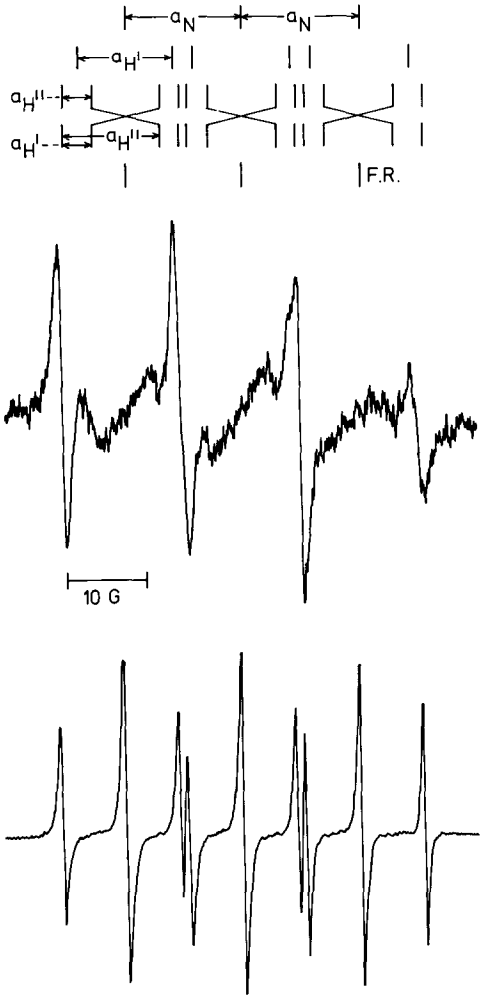


Fig.4. Viscosity dependence of the tricine radical. Tricine radical formation identical as fig.1. Upper spectrum is taken at −20°C in 30% glycerol: power 25 mW, modulation amplitude 0.02 G; gain 1.25×10^6. Lower spectrum is taken at 90°C in water: power 1 mW; modulation amplitude 0.02 G; gain 1.5×10^4. Upper 3 rows describe the high viscosity spectrum: a_N = 14.85 G; $a_{H'}$ = 12. G; $a_{H''}$ = 3.7 G. Due to rotation H′ and H″ are interchanged, (row 4), which leads in the case of fast rotation (F.R., lowest row) to an average position described by a_H = 7.85 G.

with a disappearance of the relative broad lines in fig.1 around 0°C in 30% glycerol. The explanation is that we have in fact two inequivalent proton positions with coupling constant of 12. G and 3.7 G (H′ and H″) as illustrated in rows 1–3 of the stick spectrum in fig.4. These proton positions are interchanged by rotation (row 4). Therefore at low viscosity (fast rotating) the coupling becomes equal to half the sum of the separate hyperfine coupling constants (fig.1, fig.4, lower spectrum), with ultimately at high temperature the same linewidth as the normal lines and thus double height (last row of sticks, F.R.).

At lower temperature 'exchange' broadening is observed. (Theoretically hindered rotation and exchange can be described in the same way.) While at even higher viscosity (−20°C, 30% glycerol) the separate couplings show up (fig.4, upper spectrum). The deuterated spectrum can be explained in the same way (0.57 G and 1.84 G are the couplings in this case). A special case is the residual CH^2H present, because there is a situation in which exchange between hyperfine couplings of 12. G and 3.7/6.51 G to 3.7 G and 12./6.51 G is expected.

Therefore, all the lines in the spectrum will be affected by hindered rotation, which explains the peculiar intensity distribution of the CH^2H peaks. Variation of the temperature of the deuterated tricine radical showed the expected behaviour.

4. Conclusion

The results clearly indicate that the tricine-NO radical is responsible for the observed ESR signal. Therefore care should be taken in using tricine buffer if proteins show oxidase activity, but eventually could be used to test oxidase activity in biological systems. The results further support the presence of pyruvate oxidase activity in the complexes isolated from *A. vinelandii* [2] and *E. coli*.

Acknowledgements

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