PROPERTIES OF *N*-BROMOSUCCINIMIDE MODIFIED CYTOCHROME OXIDASE

M. LANDON and R. J. MAYER

Department of Biochemistry, Medical School, University of Nottingham, University Park, Nottingham NG7 2RD, England

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1. Introduction

Previous work [1, 2] has demonstrated a direct relationship between the transfer of electrons in conduction bands and biological activity of cytochrome oxidase. This relationship was established by making microwave charge carrier Hall mobility measurements and correlating the observed electron movements in a conduction band system with the capacity of cytochrome oxidase to oxidise ferrocytochrome *c*. Further measurements of Hall mobility values indicated a close relationship between the conformation of cytochrome oxidase and electron migration in a conduction band system [2].

Experiments were therefore devised to investigate the importance of conformation to the properties of cytochrome oxidase. This communication describes chemical modifications of cytochrome oxidase and measurements of biological activity and optical properties.

2. Materials and methods

Cytochrome oxidase was prepared from beef heart mitochondria by the method of Yonetani [3]. All the experiments were carried out using a preparation in 0.1 M phosphate buffer (pH 7.4) containing 1% Tween-80 (polyoxyethylene sorbitan monooleate).

The absorbance ratio of the preparation was A_{445} (reduced)/A_{420} (oxidised) = 1.32; A_{280} (oxidised)/A_{445} (reduced) = 3.22; A_{603} (reduced)/A_{552} (reduced) = 2.57. The concentration of the enzyme and the activity were determined by the method of Yonetani [3]. The K_m for ferrocytochrome *c* was 20 μM, and the molecular activity at infinite cytochrome *c* concentration was 2,900 moles of ferrocytochrome *c* oxidised/min/mole haem a.

A stock solution of *N*-bromosuccinimide (NBS, 40 mM) was freshly prepared in distilled water and diluted prior to use to either 20 or 10 mM as required. Addition of NBS was made by pipetting the appropriate volume into a solution of cytochrome oxidase (32 μM) at room temperature with rapid mixing. Spectrophotometric and kinetic measurements were made on solutions diluted appropriately with 0.1 M phosphate buffer, pH 7.4 containing 1% Tween-80. All spectrophotometric measurements were made at least 5 min after the addition of NBS to cytochrome oxidase. No further changes in spectral properties were observed after exposure to the reagent for longer periods. Kinetic measurements were made on 5 μl samples of 0.5 μM cytochrome oxidase. No difference was observed in the kinetic parameters of NBS-modified cytochrome oxidase treated in this way and cytochrome oxidase which had been dialysed overnight against the phosphate-Tween buffer after treatment with NBS.

The degree of modification of tryptophan was calculated from the decrease in absorbance at 280 nm as described by Patchornik et al.

Spectrophotometric and kinetic measurements were made using a Unicam SP 8000 ultraviolet recording spectrophotometer with AR 25 Linear Recorder. Spectropolarmetric measurements were made with a Bendix "Polarmatic 62".

3. Results

In preliminary experiments various functional reagents were used and their effects on the kinetic, spec-

trophotometric and optical rotatory dispersion (ORD) properties of cytochrome oxidase studied. It was found that major alterations of these properties occurred with NBS at a molar ratio* some ten to twenty times less than with *N*-acetyl imidazole, acetic anhydride or succinic anhydride. The effects of NBS on cytochrome oxidase were therefore studied in more detail.

Titration of cytochrome oxidase with NBS produced the changes in biological activity shown in fig. 1.

Total loss of activity was obtained at a molar ratio of 62.5:1 at which point measurements at 280 nm [4] showed that approximately three moles of tryptophan had been modified per mole of cytochrome oxidase.

Spectrophotometric measurements are presented in fig. 2. The extent of alteration of the spectrum of cytochrome oxidase was dependent on the molar ratio of NBS to cytochrome oxidase. At the highest levels of NBS (molar ratio 62.5:1) the spectrum of the oxidised preparation showed a 2 nm red shift at 420 nm while the spectrum of the dithionite reduced preparation showed a 5 nm blue shift at 445 nm and an 8 nm blue shift at 603 nm in comparison with untreated preparations. The absorbance of the maximally modified oxidised preparation was virtually unchanged whereas the absorbance of the maximally modified reduced preparation at 440 nm and 595 nm was significantly diminished.

The progressive changes in the spectrum of cytochrome oxidase which has been modified by increasing amounts of NBS was parallelled by alterations in the ORD spectra of the preparations as shown in fig. 3.

Progressive modification of the spectrum in the Soret region of the oxidised preparation is observed with increasing molar ratio of NBS to cytochrome oxidase.

* Molar ratio is moles of *N*-bromosuccinimide/mole of cytochrome oxidase.

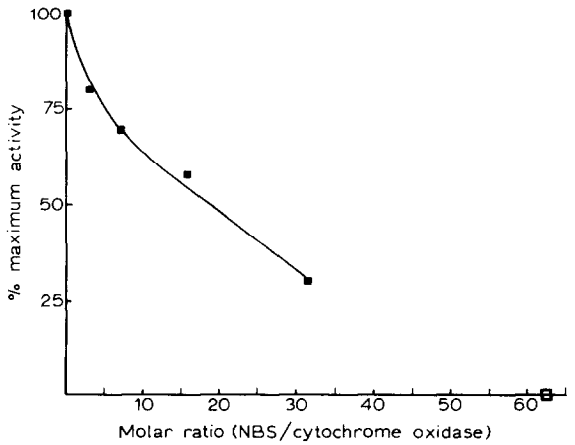

Fig. 1. The effect of NBS on the biological activity (oxidation of ferrocytochrome *c*) of cytochrome oxidase. Progressive decline in activity (■—■—■); highest molar ratio of NBS/cytochrome oxidase (□).

4. Discussion

The work of Dickerson et al. [5] on the structure of horse ferricytochrome *c* has indicated that aromatic groups have a tendency to occur in approximately parallel pairs which caused these authors to speculate on the transfer of electrons via the overlap of aromatic π-electron bonds. Furthermore such overlapping aromatic π-electron bonds in conjunction with α-helix could contribute to the formation of a conduction band system [2]. These suggested relationships can be examined in cytochrome oxidase since a direct correlation between activity and electron conduction has been established [1, 2]. The results presented in fig. 1 show decreasing activity of cytochrome oxidase with increasing amounts of NBS; 70% of the activity being lost at a molar ratio of only 31.3:1 where approx. 2 moles of tryptophan have been modified per mole of cytochrome oxidase. At a higher molar ratio (62.5:1), where no activity could be detected, approx. three moles of tryptophan were modified per mole of cytochrome oxidase. Although tryptophan oxidation was observed modification of other residues cannot be ruled out at these levels of NBS.

The effect of increasing amounts of NBS on the absorption spectra of cytochrome oxidase is shown in fig. 2. Even at the highest level of NBS the effect on the oxidised spectrum is small whereas both a significant decrease in maximum absorbance and blue shifts were observed in the dithionite-reduced spectrum. The changes in the dithionite-reduced spectrum were progressive and reflect increasing modification by NBS. The spectral changes observed after NBS treatment contrast with the spectral changes observed after treatments with large amounts of other func-

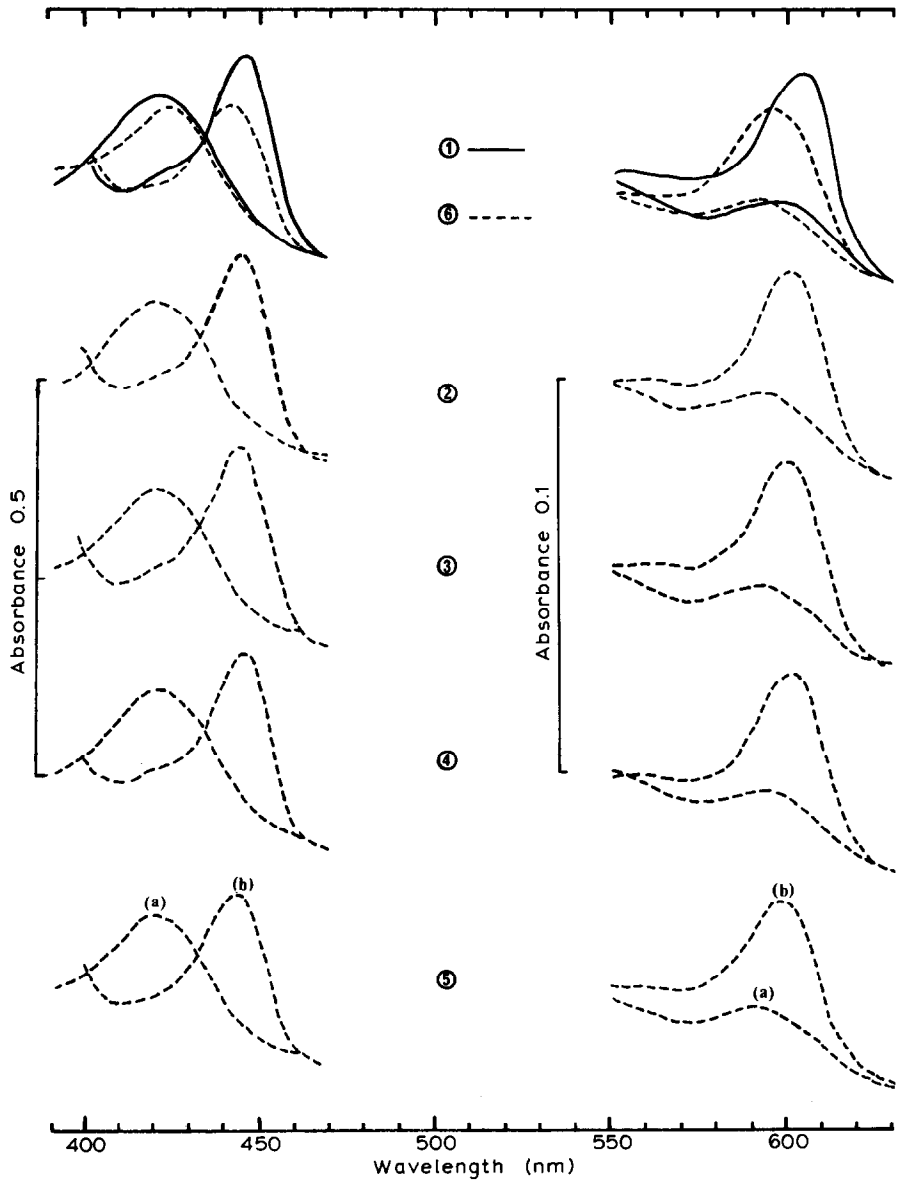

Fig. 2. Absorption spectra of cytochrome oxidase in the 390–630 nm region after treatment with NBS. (a) Oxidised preparations, (b) dithionite-reduced preparations. Unmodified preparation (——; 1), modified preparations (- - -; 2–6). Molar ratios 2, 3.9; 3, 7.9; 4, 15.6; 5, 31.3; 6, 62.5.

tional reagents (e.g. succinic anhydride) where both oxidised and dithionite-reduced spectra were drastically modified. This comparison suggests that the structural modifications of cytochrome oxidase mediated by NBS are small.

The nature of the spectral changes at the highest levels of NBS suggests little modification of the haems of cytochrome oxidase. In contrast modifications of the haem moiety of haemoglobin with abolition of the Soret band have been observed at high levels of NBS [6].

These modifications of cytochrome oxidase by NBS are further reflected by alterations in the ORD spectra as shown in fig. 3. Since haem a in detergent

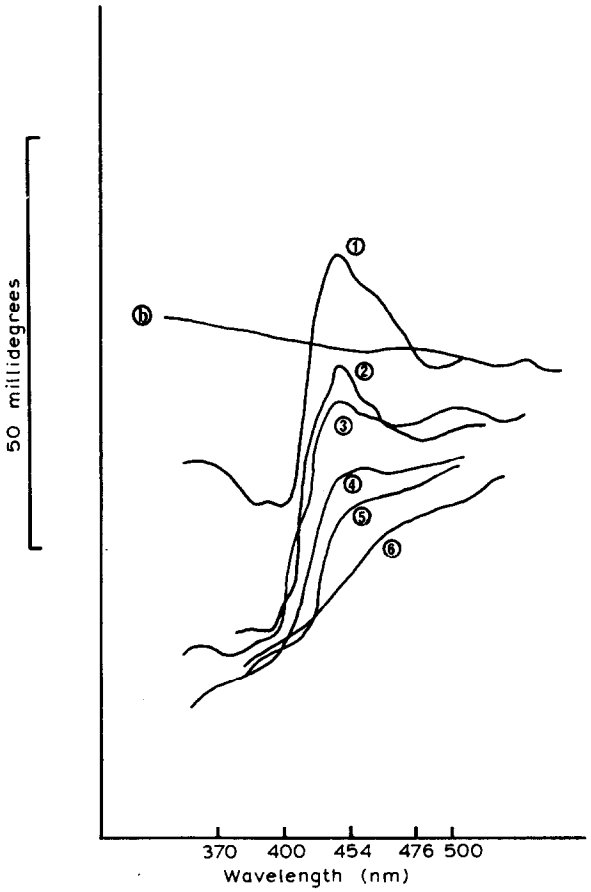

Fig. 3. Optical rotatory dispersion spectra of cytochrome oxidase in the Soret region after treatment with NBS. Baseline (b); 1–6, molar ratios as in fig. 2.

solution is optically inactive [7] and the haem moieties in cytochrome oxidase after NBS treatment are not modified the progressive alterations in ORD observed must be the result of changes in haem protein interactions.

If the interaction between aromatic residues is related to electron transfer in cytochrome oxidase then chemical modification of these residues should affect the electron transfer process within the complex. The evidence presented here indicates that the changes mediated by NBS severely disrupt the biological activity of cytochrome oxidase.

Acknowledgement

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