Journal of Chromatography, 317 (1984) 407–412
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 430

DETERMINATION OF QUINONOID DIHYDROBIOPTERIN BY LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

CRAIG E. LUNTE* and PETER T. KISSINGER
Department of Chemistry, Purdue University, West Lafayette, IN 47907 (U.S.A.)

SUMMARY

Tetrahydrobiopterin (BH4) is a required cofactor for the hydroxylation of the amino acids phenylalanine, tyrosine and tryptophan. Quinonoid dihydrobiopterin (q-BH2) is the natural product of the oxidation of BH4 in these enzymatic reactions. This report describes a method based on liquid chromatography and electrochemical detection for the determination of q-BH2 in synthetic and biological samples. This methodology is used to study the electrochemistry of q-BH2 formed by oxidation of BH4 and in the hydroxylation of phenylalanine by phenylalanine hydroxylase.

INTRODUCTION

The reduced pterin, l-*erythro*-5,6,7,8-tetrahydrobiopterin (BH4), has been shown to be a required cofactor for the enzymatic hydroxylation of the amino acids phenylalanine, tyrosine and tryptophan[1]. In these reactions, BH4 is oxidized to a labile dihydrobiopterin (BH2). This species is either reduced back to BH4 or tautomerizes to 7,8-BH2[2]. Although the general reaction scheme,

$$AAH + BH4 + O_2 \rightarrow AAOH + q\text{-}BH2 + H_2O \quad (1)$$

$$q\text{-}BH2 \rightarrow BH2 \quad (2)$$

where AAH is the amino acid and AAOH its hydroxylated form, is well accepted, the actual structure of the BH2 intermediate has yet to be established. It has been generally assumed that the labile dihydrobiopterin has a quinonoid structure[3,4].

Quinonoid dihydrobiopterin (q-BH2) has been determined by liquid chromatography with either UV-adsorption[5] or electrochemical[6] detection. In both cases q-BH2 was generated by the chemical oxidation of BH4 with ferricyanide. In our report, the detection of q-BH2 as an enzymatic oxidation product of BH4 is demonstrated. Liquid chromatography with electrochemical detection is capable of resolving and detecting all of the oxidation states of biopterin in an enzymatic incubation. In addition, the electrochemistry of q-BH2 can be studied. A dual-electrode detector is used to generate the labile q-BH2 from BH4 and to study the electrochemistry at the downstream electrode.

0021-9673/84/$03.00

EXPERIMENTAL

Materials

Biopterin was purchased from Calbiochem-Behring (La Jolla, Ca, U.S.A.). L-Phenylalanine and L-tyrosine were obtained from Sigma (St. Louis, MO, U.S.A.). Octyl sodium sulfate was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). BH4 and 7,8-BH2 were prepared as described previously[7].

Apparatus

The chromatographic system consisted of an Altex 110 pump and a Rheodyne 7010 injection valve with a 20-μl sample loop. A Brownlee MPLC RP-18 5-μm column (10 cm × 4.6 mm) and dual LC-4B amperometric detectors (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with glassy carbon working electrodes and a Ag/AgCl reference electrode were used.

Liquid chromatography

The mobile phase was 3 m*M* octyl sodium sulfate in a 0.1 *M* sodium phosphate buffer (pH 2.5), containing 15% methanol (v/v). The mobile phase was prepared from distilled, deionized water and glass distilled methanol and was filtered through a 0.22-μm filter (Millipore, Milford, MA, U.S.A.) prior to use. Oxygen was removed from the mobile phase by continuously purging with nitrogen while maintaining the reservoir at 40°C. A flow-rate of 2.0 ml/min was used in all experiments.

Metal ions are known to be adsorbed on reversed phase columns and can react with pterins[8]. To avoid any problems from adsorbed metals, after each experiment involving chemical oxidation of BH4, a column cleaning procedure was followed, as suggested by Haavik and Flatmark[5]. Several injections were made of a 1.0 *M* sodium dithionite solution followed by flushing the column with a 0.1 *M* EDTA solution in 0.1 *M* sodium phosphate buffer (pH 6.8). This was followed by re-equilibration of the column with the mobile phase.

Chemical oxidation

Potassium ferricyanide was used to oxidize BH4 to q-BH2. At a mole ratio ferricyanide:BH4 of 2:1, oxidation to q-BH2 was complete in a few seconds. The ferricyanide–q-BH2 mixture was directly injected into the chromatographic column.

Enzymatic oxidation

Mice were killed by decapitation, and their livers were removed and homogenized. Phenylalanine hydroxylase was isolated by precipitation with ammonium sulfate (between 35% and 45% saturation). The precipitate was dissolved in 0.01 *M* sodium phosphate buffer (pH 7.5) and purified on a DEAE-Sephadex column, as described by Shiman *et al.*[9].

The incubation mixture was as described previously[10], except that BH4 was employed as the pterin cofactor. The incubation was allowed to proceed for 2 min, after which a 200-μl aliquot was removed and diluted to 2.0 ml with 0.1 *M* sodium phosphate buffer (pH 2.5). This sample was deoxygenated for 2 min, and then 20 μl was injected into the chromatographic column.

RESULTS

Voltammetry

Because q-BH2 is not stable in solution, tautomerizing to 7,8-BH2, it is not possible to obtain voltammetric data on a standard solution. However, hydrodynamic voltammetry can be performed by using the dual-electrode amperometric detectors in series. In this method, a standard solution of BH4 is oxidized at the upstream electrode. This oxidation produces q-BH2, which can be studied electrochemically at the downstream electrode. This technique has been demonstrated for quinonoid dihydro-6-methylpterin[11].

Fig. 1 shows the hydrodynamic voltammogram of q-BH2 obtained with the dual-electrode detector. A standard solution of BH4 was repetively injected while the upstream electrode was kept at a constant oxidizing potential. This generates a reproducible amount of q-BH2 which is independent of the potential applied to the downstream electrode. The hydrodynamic voltammogram of q-BH2 is then generated by varying the potential of the downstream electrode and plotting the ratio of downstream response to upstream response *versus* applied potential at the downstream electrode. Use of the ratio of downstream response to upstream response normalizes the hydrodynamic voltammogram for any change in the upstream electrode.

BH4 and 7,8-BH2 are relatively stable in solution. Therefore, hydrodynamic voltammetry can be performed directly with standard solutions of these species, as

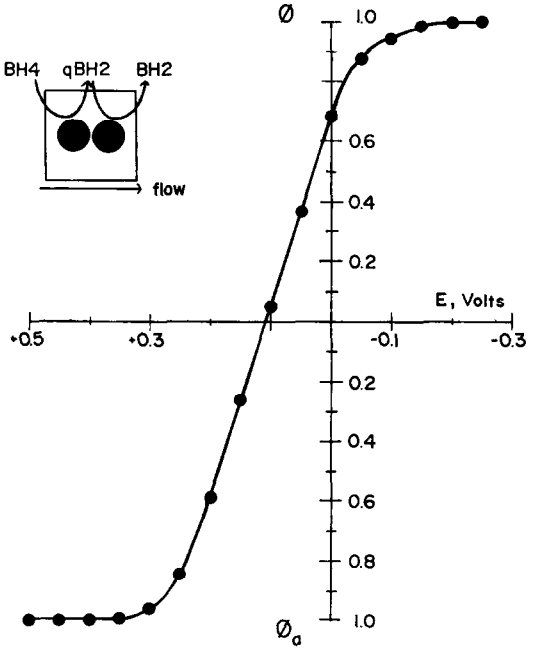

Fig. 1. Hydrodynamic voltammogram of q-BH2 obtained by the oxidation of BH4 upstream. Ø is the normalized current response.

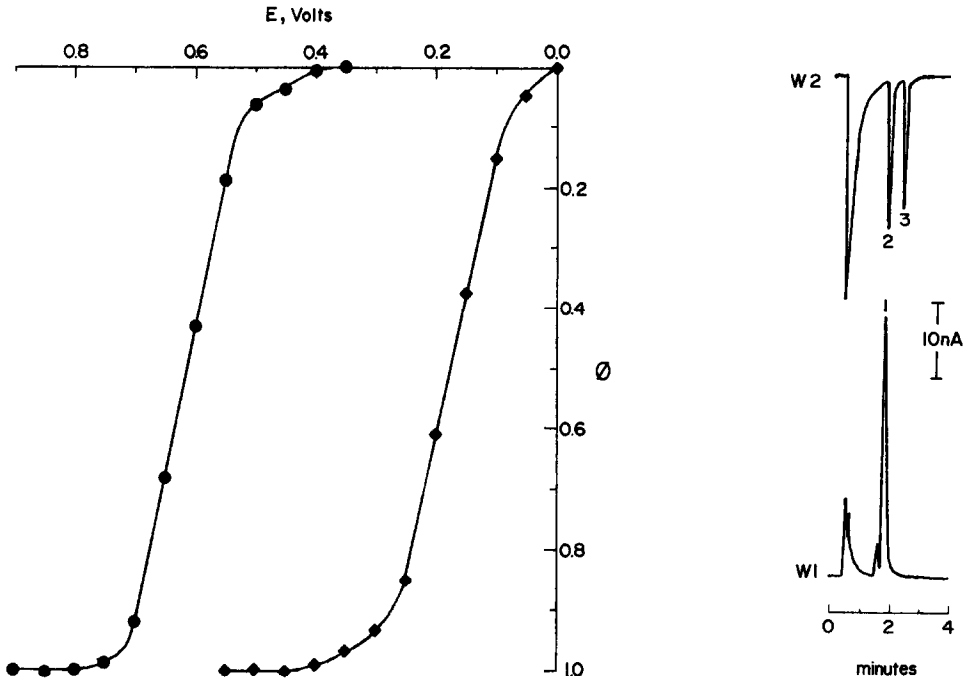

Fig. 2. Hydrodynamic voltammograms of BH4 (◆) and 7,8-BH2 (●).

Fig. 3. Separation of the three biopterin species. Chromatographic conditions: 3 m*M* octyl sodium sulfate in a 0.1 *M* phosphate buffer (pH 2.5), containing 15% methanol (v/v). Electrode potentials: W1 = −300 mV, W2 = +900 mV. Peak identities: 1 = q-BH2; 2 = BH4; 3 = 7,8-BH2.

illustrated in Fig. 2. This provides voltammetric information for all of the pterin species of interest.

Chromatography

The separation of BH4, 7,8-BH2, and q-BH2 is shown in Fig. 3. Being similar in structure, BH4 and q-BH2 were not resolved chromatographically. However, because the two compounds differ in electrochemical reactivity, BH4 being easily oxidized and q-BH2 being easily reduced, they can be distinguished by the electrochemical detectors.

The standard solution used to generate the chromatogram in Fig. 3 was a mixture of BH4 and 7,8-BH2 which was partially oxidized by ferricyanide. The identity of the q-BH2 peak was confirmed by voltammetric characterization, the standard being produced in the series dual-electrode experiment, as has been described for 6-methylpterin[11].

Characterization of BH4 oxidation products

Fig. 4 shows chromatograms obtained 15 sec and 15 min after oxidation of a BH4 solution by ferricyanide. These chromatograms show formation of an initial labile oxidation product, which is converted to a relatively stable final product. Based

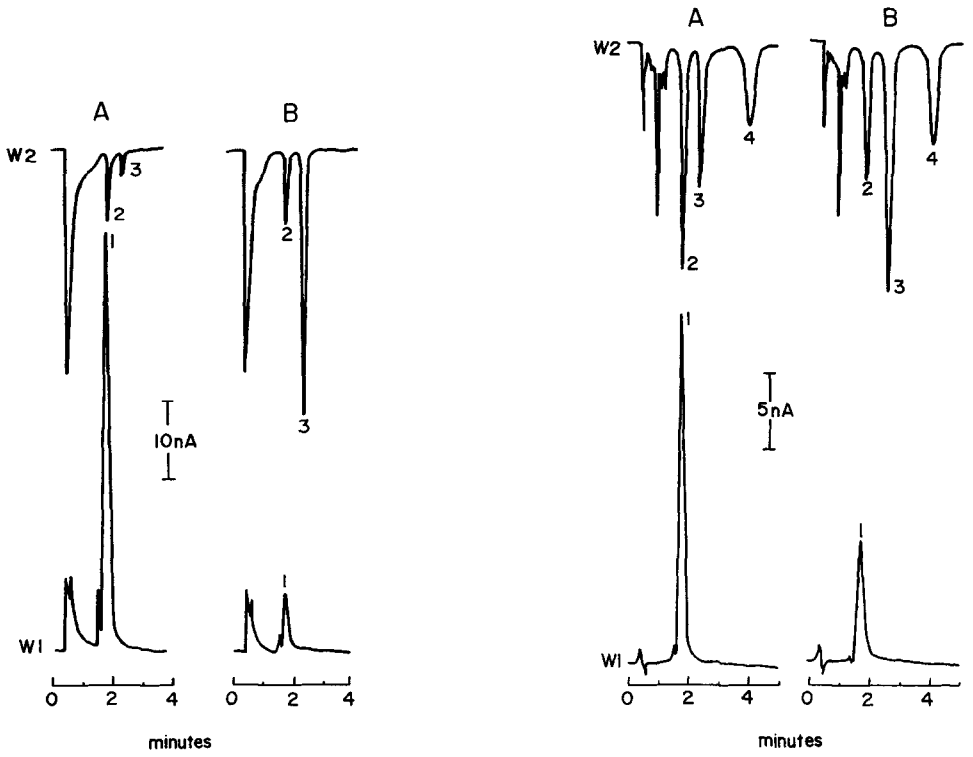

Fig. 4. Chromatograms of the products of oxidation of BH4 by ferricyanide. (A) 15 sec; (B) 15 min. Peak identities and chromatographic conditions as in Fig. 3.

Fig. 5. Chromatograms of the products of oxidation of BH4 by phenylalanine hydroxylase. (A) 2 min; (B) 15 min. Peak identities and chromatographic conditions as in Fig. 3.

on voltammetric data, the initial product has been identified as q-BH2. The final product has been identified as 7,8-BH2, based on both voltammetric data and retention data.

Chromatograms obtained 2 min and 15 min after incubation of BH4 with phenylalanine and phenylalanine hydroxylase are shown in Fig. 5. The same products were obtained through this enzymatic oxidation as were found for the chemical oxidation procedure. That the oxidation indeed proceeded, at least partially, through an enzymatic path is shown by the appearance of tyrosine in the incubation mixture. In the absence of phenylalanine hydroxylase no tyrosine is formed.

DISCUSSION

q-BH2 has previously been detected by liquid chromatography with UV-absorption detection. Haavik and Flatmark[5] found it difficult to resolve q-BH2 and BH4 chromatographically, a result confirmed by this work. They were able to distinguish these two species partially by detection at two wavelengths, but since the spectra overlap, this approach is not completely satisfactory. We have shown that this problem can be circumvented by the use of a dual-electrode amperometric de-

tector. Because the q-BH2 is easily reduced while the BH4 is easily oxidized, these species can be separately detected electrochemically by a judicious choice of operating potentials.

Due to the lability of q-BH2, it is impossible to perform voltammetry on a standard solution. Using the dual-electrode detectors in series, q-BH2 can be generated at the upstream electrode and its electrochemistry studied at the downstream electrode. This technique is analogous to rotating ring/disk electrode voltammetry. The liquid chromatographic experiment offers advantages over bulk techniques by presenting a single component to the electrodes and requiring smaller sample volumes.

Finally, this report demonstrates the production of q-BH2 through the enzymatic oxidation of BH4. These results indicate that investigating the electrochemistry of BH4 at electrodes is a valid method of probing the enzymatic reaction. This method should be capable of detecting q-BH2 in biological samples, such as brain and liver tissue, providing the sample preparation time can be kept to a minimum to avoid tautomerization.

REFERENCES

1 S. Kaufman and D. B. Fisher, in O. Hayaishi (Editor), *Molecular Mechanisms of Oxygen Activation,* Academic Press, New York, 1974, pp. 285.
2 K. H. Nielsen, V. Simosen and K. E. Lind, *Eur. J. Biochem.*, 9 (1969) 497.
3 S. Kaufman, *J. Biol. Chem.*, 236 (1961) 804.
4 S. Kaufman, *J. Biol. Chem.*, 239 (1964) 332.
5 J. Haavik and T. Flatmark, *J. Chromatogr.*, 257 (1983) 361.
6 C. E. Lunte and P. T. Kissinger, *Anal. Chim. Acta,* 158 (1984) 33.
7 C. E. Lunte and P. T. Kissinger, *Anal. Chem.*, 55 (1983) 1458.
8 D. J. Vonderschmitt and K. G. Scrimgeour, *Biochem. Biophys. Res. Commun.*, 28 (1967) 302.
9 R. Shiman, D. W. Gray and A. Pater, *J. Biol. Chem.*, 254 (1979) 11,300.
10 C. E. Lunte and P. T. Kissinger, *Anal. Biochem.*, 139 (1984) 468.
11 C. E. Lunte and P. T. Kissinger, *Anal. Chem.*, 56 (1984) 658.