Reaction of cyanide with cytochrome aa_3 in isolated perfused rat head *in situ*

Khalil KARIMAN and D. Scott BURKHART

Department of Medicine, Duke University Medical Center, P.O. Box 3848, Durham, NC 27710, U.S.A.

The reaction of cyanide with cytochrome aa_3 in intact mitochondria is known to differ significantly from the reaction with the isolated enzyme. To examine the cyanide reaction with cytochrome aa_3 *in situ*, we studied the spectral characteristics and the reaction kinetics of cyanide with reduced brain cytochrome aa_3 in an isolated perfused rat head preparation. Anaesthetized rats underwent bilateral carotid-arterial cannulation. The head (skull intact, muscle removed) was perfused with a crystalloid solution containing $\text{Na}_2\text{S}_2\text{O}_4$, and the animal was then decapitated. By means of reflectance spectrophotometry the reaction of cyanide with cytochrome aa_3 was continuously monitored with the use of the 590 nm–575 nm, 610 nm–575 nm and 590 nm–610 nm wavelength pairs. We found that: (a) the kinetics of the absorbance change at 590 nm and 610 nm were similar, with almost identical apparent rate constants, suggesting that these spectral changes are the results of the formation of a single complex; (b) the difference spectrum obtained on addition of cyanide to the fully reduced preparation showed a peak at 588 nm and a trough at 610 nm, consistent with spectral characteristics of the cyanide–ferrocycytochrome aa_3 complex in isolated enzyme and isolated mitochondria *in vitro*; this observation underscores the accuracy of monitoring the effects of inhibitors of mitochondrial function on cytochrome redox reactions *in situ*; (c) the half-maximal ($K_{0.5}$) effect was approx. 50 μM , significantly lower than that *in vitro*. The lower apparent $K_{0.5}$ for cyanide in this preparation *in situ* may be due to a difference in the pH of the two systems. This approach provides the means to study the inhibitors of mitochondrial function in intact brain under a physiological environment.

INTRODUCTION

The site of action of cyanide on cytochrome oxidase was first studied by Keilin & Hartree (1939). They found that both the reduced and the oxidized forms of the oxidase were capable of cyanide binding, and that the ferric form of cytochrome a_3 is the form ‘stabilized’ by cyanide. Later, Chance (1952) concluded that the reduced oxidase form must be more vulnerable to the action of this inhibitor.

The reaction of cyanide with cytochrome oxidase has been fully studied by many investigators *in vitro* (Gibson & Greenwood, 1963; Horie & Morrison, 1963; Yonetani & Ray, 1965; Vanneste, 1966; Antonini *et al.*, 1971; Erecinska *et al.*, 1972; Nicholls *et al.*, 1972; Van Buuren *et al.*, 1972a,b; Wilson *et al.*, 1972; Nicholls & Chance, 1974; Yoshikawa & Orii, 1974). However, the spectral and kinetic characteristics of this reaction in intact organs are not known. It is not clear whether one can extrapolate from these observations made *in vitro* to the conditions existing under physiological conditions. In fact, the reaction of cyanide with cytochrome oxidase can differ significantly depending on the preparation used. For instance, Van Buuren *et al.* (1972a,b) showed that equilibrium between cyanide and cytochrome aa_3 can be reached more rapidly during active turnover of the enzyme. More relevant, perhaps, was the observation made by Wilson and co-workers (Wilson *et al.*, 1972; Erecinska *et al.*, 1972) with intact mitochondria. Reaction of cyanide with oxidized cytochrome aa_3 , in particular, was found by these investigators to be first-order in intact mitochondria, whereas it is known to deviate from first-order in the isolated enzyme (Chance,

1952; Gibson & Greenwood, 1963; Van Buuren *et al.*, 1972b). Similarly, the rate of reaction of cyanide with oxidized cytochrome aa_3 was found to be much faster in the intact mitochondria than in the isolated enzyme.

With the use of organ spectrophotometry, the redox reactions of mitochondrial cytochromes can be reliably monitored in intact tissue in their physiological environment *in situ* (Chance, 1951; Rosenthal *et al.*, 1976; Sies & Brauser, 1980; Kariman *et al.*, 1983). In an isolated perfused rat head model (skull intact) we have recently examined the electron-transfer reactions (K. Kariman & D. S. Burkhardt, unpublished work) and cell bioenergetics (K. Kariman, B. Chance, D. S. Burkhardt, L. Bolinger, E. Donlon & A. C. McLaughlin, unpublished work) *in situ*. Inhibitors of the respiratory chain can now be used to study mitochondrial function in this model. In the present studies the reaction of cyanide with cytochrome oxidase was examined. We chose here to study the reduced cytochrome oxidase since, in contrast with the situation *in vitro*, full oxidation of this enzyme *in situ* cannot be attained by ordinary means and would require the use of hyperbaric oxygen. This has been related to the presence of a possible diffusion barrier *in vivo* (Kariman & Burkhardt, 1985). These techniques evolving from the study of isolated mitochondria *in vitro* and perfused organ *in situ* to living laboratory animals *in vivo* should allow us to follow continuously the nature of cell biochemistry under a physiological environment.

MATERIALS AND METHODS

A more detailed description of the methods has been reported elsewhere (Kariman & Burkhardt, 1985).

Surgical preparation

Male Wistar rats were anaesthetized with pentobarbital (Nembutal; 50 mg/kg, intraperitoneally) and underwent a tracheostomy as well as femoral-venous catheterization. After a midline scalp incision, the underlying fascia, muscle and periosteum were carefully dissected and scraped free from the skull surface. The common carotid arteries were then identified and dissected free from the carotid sheath. A hypothermic ($23 \pm 1^\circ\text{C}$) infusion was initiated immediately after the right and left common carotid cannulations. The time required for cannulation of each carotid artery, during which circulation was interrupted, was generally less than 2 min. Just before the actual cannulation 150–200 i.u. of sodium heparin was administered intravenously; 2.5 mg of sodium pentobarbital was periodically administered at this point. With the head continuously perfused, the animal was then decapitated and the head securely mounted in a stereotaxic holder for optical monitoring. The muscle tissues surrounding the skull surface were finally repacked with cotton-filled sponges and the optical probes were positioned.

Head perfusion

By using a roller pump, the head was perfused with a crystalloid solution pre-equilibrated with O_2/CO_2 (19:1). This was a Krebs–Henseleit solution to which MgCl_2 (0.043%) and hydroxyethyl-starch (4%, w/v) were added. The CO_2 and pH of the solution were maintained at approx. 36 mmHg and 7.40 ± 0.05 (s.d.) respectively. This perfusate solution proved to be satisfactory in maintaining the preparation free of oedema for a minimum of several hours. A simple oxygenator was made by using a 10 ml pipette. Relatively rapid changes in gas concentrations of the solution could be made by utilizing this system. An O_2 analyser continuously recorded the O_2 concentration of the perfusate, and the perfusion pressure was continuously monitored via a Statham pressure transducer.

Reflectance spectrophotometric monitoring

A quadruple-beam spectrophotometer with a single light source for four monochromators was used. Optical coupling rods of diameter 6.2 mm ($\frac{1}{4}$ in) were used for the light bundle and photomultiplier. The light rod was located below the left temporal ridge and situated flush against the skull surface, while the photomultiplier rod was located slightly to the left, off centre of the sagittal suture. A small amount of optical coupling gel was placed on the end of the photomultiplier rod and then positioned against the skull surface and sealed with a small 'O' ring. This particular positioning ensures efficient interaction of the light with the tissue, thus providing a higher concentration of cytochrome available for monitoring. By using the dual-wavelength principle of Chance (1951) the reaction of cyanide with reduced cytochrome oxidase was continuously monitored with the 590 nm–575 nm, 610 nm–575 nm and 590 nm–610 nm wavelength pairs. In the dual-wavelength technique, one of the two monochromators provides light at the absorption peak of a component chosen for study, with the other at a close indifferent wavelength. The latter serves as a reference to compensate for light-scattering changes. The two light beams are alternatively presented to a single biological preparation and the emerging light is measured by a

photomultiplier and recorded as their difference. This mode makes it possible to monitor kinetically the absorption changes of a single component in the presence of non-specific changes in scattering.

The difference spectrum was determined by subtracting the previous scan from the present scan at 2 nm increments for the 500–620 nm wavelength range. Two monochromators were commonly used, with one adjusted to 575 nm (reference) and the other (sample) used for scanning. Before the scanning, the reference and sample monochromators were set at 575 nm and the intensity of light transmission was adjusted to provide a 0.5 V signal for both channels. In the absorption mode, scans were taken with the sample monochromator. Each wavelength was therefore referenced to 575 nm. A narrow bandwidth of 3–4 nm was used for both sample and reference wavelengths.

Chemicals

KCN solutions were freshly made in perfusate pre-equilibrated with N_2/CO_2 (19:1). In a series of experiments the cyanide concentration required to induce maximal spectral changes was first determined. Experiments were then conducted to determine the equilibrium dissociation constant as well as the apparent rate constant.

The experimental protocol was as follows. After perfusion of the organ with approx. 50 ml of perfusate solution containing small amounts of $\text{Na}_2\text{S}_2\text{O}_4$ a baseline scan was obtained, and various concentrations of KCN were then added to the perfusate solution. The second scan was recorded and the experiment was terminated by interrupting the perfusion.

RESULTS

Fig. 1 depicts the formation of the cyanide–ferrocyclochrome aa_3 complex monitored at 590 nm–575 nm and 610 nm–575 nm. This Figure shows that the kinetics of the absorbance change at 590 nm and 610 nm are almost identical. The apparent rate constant (K_{obs}) for the absorbance change at these two wavelengths was calculated to be 0.007 s^{-1} for 1 mM-cyanide in this experiment. Note the stability of the reference (575 nm) signal.

The addition of cyanide to a fully reduced isolated perfused rat head preparation induces the absorbance change shown in Fig. 2. The change represents absorption decreases near 610 nm and 568 nm and absorption increase near 588 nm.

Fig. 3 depicts the rate of reaction of cyanide with cytochrome aa_3 monitored continuously at 590 nm–610 nm. The half-time for the reaction of 5 mM concentration of cyanide used in this experiment was about 1 min. This concentration of cyanide is in approx. 10-fold excess of the concentrations required to achieve a half-saturation state. The absorbance change induced by the addition of such high concentrations of cyanide to the preparation in which the cytochromes are highly reduced is kinetically pseudo-first-order. As shown in Fig. 4, the natural logarithm of the ratio of the absorbance change that occurs from $t = 0$ to $t = \text{infinity}$ (A) to the absorbance change that occurs between time t and infinity ($A - X$) is a linear function of time over the period that can be readily measured (0–90% completion). Under these experimental conditions the apparent rate constant

Fig. 1. Example of formation of cyanide-ferrocytochrome aa_3 complex monitored at 590 nm–575 nm and 610 nm–575 nm in isolated perfused rat head (skull intact, muscle removed)

A crystalloid solution (see the text) pre-equilibrated with N_2/CO_2 (19:1) and containing a small amount of $Na_2S_2O_4$ was used as perfusate. Cyanide at concentrations indicated (arrows) was then added to the perfusate solution. Note the stability of the reference (575 nm) signal.

Fig. 2. Spectral change induced by adding cyanide to the reduced cytochrome oxidase in isolated perfused rat head

The difference spectrum represents dithionite reduced preparation + 7.5 μM -cyanide minus dithionite-reduced preparation. With the use of the dual-wavelength principle, each wavelength was referenced to 575 nm. Other conditions are described in Fig. 1 legend.

($K_{obs.}$) determined from the slope of the line was $0.018 s^{-1}$.

The cyanide-concentration-dependence of the reaction with reduced brain preparation monitored at 590 nm–610 nm is shown in Fig. 5. At this wavelength pair, a plot of the reciprocal of the absorbance change against the reciprocal of cyanide concentrations (Fig. 6) is linear, and the half-maximal ($K_{0.5}$) effect is obtained with approx. 50 μM -cyanide.

Fig. 3. Rate of reaction of 5 mM-cyanide with reduced cytochrome aa_3 in isolated perfused rat head preparation monitored at 590 nm–610 nm

Other conditions are described in Fig. 1 legend.

DISCUSSION

Except for the K_D , the reaction of cyanide with reduced cytochrome oxidase in the isolated perfused rat brain preparation *in situ* reported here was similar to what has been described for the isolated enzyme as well as for intact mitochondria *in vitro*. This model now provides an opportunity to study the means of displacing the cyanide bound to cytochrome aa_3 and therefore reversing its toxicity.

The difference spectrum obtained on addition of cyanide to the fully reduced preparation showed a peak at 588 nm and a trough at 610 nm. This is very similar to spectral changes obtained by addition of cyanide to ferrocytochrome aa_3 reported in the literature (Horie &

Fig. 4. Pseudo-first-order reaction of 5 mM-cyanide with reduced cytochrome aa_3 for isolated perfused rat head preparation of the experiment shown in Fig. 3

Absorbance change occurring from $t = 0$ to $t = \infty$ is identified as A , and the absorbance change that occurs at time t is identified as X . Other conditions are described in Fig. 1 legend.

Morrison, 1963; Yonetani & Ray, 1965; Vanneste, 1966; Antonini *et al.*, 1971; Van Buuren *et al.*, 1972b; Wilson *et al.*, 1972). For instance, with isolated enzyme Van Buuren *et al.* (1972b) showed a peak at 587 nm and trough at 611 nm. Similarly, with intact mitochondria Wilson *et al.* (1972) reported a peak and a trough at 591 and 612 nm respectively after addition of cyanide to the fully reduced preparation. Similarities between these preparations *in vitro* and our preparation *in situ* further confirm the reliability of spectrophotometric techniques in examining the function of mitochondria in their physiological environment in the presence of inhibitors.

In isolated cytochrome oxidase, as well as in intact mitochondria, the rate of the cyanide reaction with the reduced cytochrome oxidase occurs too rapidly to be measured without the use of rapid-mixing technique. In the isolated perfused preparation, on the other hand, the formation of the cyanide-ferrocyanide aa_3 complex is slow and provides an opportunity to observe this reaction continuously simultaneously at different wavelengths. As shown in Fig. 1, the 590 nm peak and 610 nm

Fig. 6. Concentration-dependence of the cyanide-induced spectral change in reduced cytochrome aa_3 in isolated perfused rat head preparation of the experiment shown in Fig. 5

The reciprocal of the absorbance change (590 nm–610 nm) is plotted on the ordinate and the reciprocal of the cyanide concentration on the abscissa; $K_{0.5} = 50 \mu\text{M}$.

trough develop at the same rate, indicating that the spectral changes are the results of the formation of a single complex (cyanide-ferrocyanide aa_3). However, one cannot rule out the possibility of two different complexes with identical kinetics within the range of experimental error. The lower rate of the cyanide reaction with reduced cytochrome aa_3 observed in isolated perfused head preparation, when compared with that in isolated cytochrome oxidase or intact mitochondria, might be due to a system proceeding through a series of equilibrium states due to diffusional barriers. However, differences in organism, pH and temperature that may exist between the two systems would certainly affect this rate.

As shown in Fig. 6, the plot of the reciprocal of the absorbance change (590 nm–610 nm) against the reciprocal of cyanide concentrations was linear, with the half-maximal ($K_{0.5}$) effect of approx. $50 \mu\text{M}$ -cyanide. It is noteworthy that with the isolated enzyme Van Buuren *et al.* (1972a) reported a K_D of cyanide of approx. $100 \mu\text{M}$ for complex-formation with the ferrous enzyme. However, with intact mitochondria in which the cytochromes were

Fig. 5. Example of the cyanide-concentration-dependent reaction with reduced cytochrome aa_3 in brain preparation monitored at 590 nm–610 nm

Other conditions are described in Fig. 1 legend.

highly reduced Wilson *et al.* (1972) reported a dissociation constant for cyanide of approx. 300 μM . In our system the cyanide concentration in the mitochondria is not known, making it difficult to distinguish the value of k_D from other values reported. We are, however, inclined to attribute this to the difference in pH in the intact organ. We did not measure the intracellular pH (pH_i) in these experiments.

To our knowledge the spectral characteristics and reaction kinetics of the cyanide-cytochrome aa_3 complex *in situ* have not previously been reported. These studies are the first step in future research efforts investigating the neurotoxicity of cyanide *in vivo* and especially the means of displacing cyanide bound to cytochrome aa_3 . As emphasized by these experiments, when cyanide binds to cytochrome aa_3 it significantly displaces and distorts the absorptive characteristics of cytochrome aa_3 . Therefore the wavelength pair that is normally used to monitor cytochrome aa_3 (namely 605 nm–620 nm) may have questionable relevance to the cyanide-cytochrome aa_3 complex, which has a peak near 590 nm and a trough at 610 nm. For this reason, we consider that it would be useful to compare the 590 nm–610 nm pair with the 605 nm–620 nm pair, which has been employed in studying the ameliorating effects of $\text{Na}_2\text{S}_2\text{O}_3$ and NaNO_3 on cyanide neurotoxicity (Piantadosi *et al.*, 1983). Finally, a stable reference signal is of utmost importance for monitoring of cytochrome redox reactions *in situ* by the dual-wavelength principle. It seems that the 575 nm wavelength can be reliably used as reference for the monitoring of the cyanide-ferrocytochrome aa_3 complex at different wavelengths.

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