TEMPERATURE-JUMP CIRCULAR DICHROISM: OBSERVATION OF CHIROPTICAL RELAXATION PROCESSES AT MILLISECOND TIME RESOLUTION

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Temperature-jump circular dichroism, a new relaxation technique, has been used to study the interaction of liver alcohol dehydrogenase with the chromophoric inhibitor, auramine O. While a single relaxation, τ = 250 μs, is observed in transmission parameters, the simultaneous observation of transient CD indicates two processes, $\tau_1 < 300$ μs and τ_2 = 200 ms. The slower process is interpreted as a relaxation between two conformational states of the enzyme-inhibitor complex which are present in solution. These differ substantially in their symmetry properties. Temperature-jump circular dichroism provides information on rates of conformational changes in biomolecular structures which is not accessible by other spectroscopic methods.

INTRODUCTION

We wish to report the observation of transient circular dichroism and absorbance changes following the single-shot temperature-jump relaxation of an enzyme-ligand equilibrium system. The measurement of transient chiroptical parameters has previously been reported, following stopped flow mixing for circular dichroism (1a,b;2) and optical rotation (3a,b;4) and for CD following flash photolysis (5). Temperature jump measurements using absorbance (6), emission (7) and scattering parameters (8) have hitherto been unable to yield direct information about the rates of symmetry-linked transient processes involved in conformational changes of biological molecules. Such processes are fundamental to control mechanisms in a variety of allosteric and related macromolecular systems (e.g. 9,10).

The enzyme liver alcohol dehydrogenase (EC 1.1.1.1) binds the chromophoric inhibitor auramine O, a diphenylmethane dye with $K_D \sim 10^{-5}$M at 25°C

Abbreviations used: LADH, liver alcohol dehydrogenase: AuO, auramine O: CD, circular dichroism: TX, transmission.

(11). The complex LADH.AuO develops intense circular dichroism which has been interpreted as involving a single highly chiral form of the inhibitor (12).

The equilibrium mixture was found to be temperature-sensitive and suitable for a study by temperature-jump CD.

EXPERIMENTAL

The experimental system consisted of a commercial temperature jump cell, path length 0.7 cm; total volume 1 ml with gold electrodes and a detection system for simultaneous observation of CD and transmission (TX) developed from that previously described for rapid Stopped Flow Circular Dichroism (SFCD), Ref. 2. Additional sensitivity was obtained by using (i) improved detector matching and low noise cascode amplification, (ii) broader band-width filtering for improved time resolution, (iii) fast high-precision two-quadrant analog division and (iv) precision source-ratioing in the TX signal channel (13). This improved system will be described elsewhere (14). The light source was a high intensity mercury arc (100 watt; Hanovia UV-100) with interference filter to select the 436 nm emission line. CD units are given as ΔA (in absorbance units) = $\Delta\epsilon.c.l$, where c is molar concentration and l, path length in cm.

The enzyme, horse-liver alcohol dehydrogenase was obtained from Boehringer Corporation as a crystalline suspension in 0.2 M phosphate buffer, and was dialysed exhaustively before use. Auramine O was obtained from Hopkin & Williams Ltd. and was recrystallised from 0.02 M sodium chloride to give $\epsilon_{430} = 4.41 \times 10^4\ M^{-1}cm^{-1}$ (11).

RESULTS AND DISCUSSION

The relaxation processes were observed on an equilibrium mixture of LADH 50 μN and AuO 36 μN, giving $\sim$25 μN LADH.AuO complex. The buffer, 0.1 M phosphate pH 7.4, was used as supporting electrolyte. At 15°C, the circular dichroism is $\Delta A = -260 \times 10^{-6}$, absorbance A = 0.75, i.e. T = 17.9% at λ = 436 nm. Discharge of a 50 nF capacitor at 20 kV into the solution (resistance $\sim$100 ohms; discharge energy $\sim$10 J) gave a measured temperature jump of $\sim$5°C in 2.5 μsec. The relaxation of CD and TX properties are shown in Fig 1A & B for multiple superimposed shots, and for a single shot (15 kV) in Fig 1C. In TX, a single transient process was observed, $\tau < 300$ μsec, faster than the time resolution of the transmission channel of the combined CD/TX detection system. In CD, two transient processes are observed, one rapid $\tau_1 < 300$ μs, faster than the time resolution of the CD channel, oppositely signed and one slower, τ_2 = 200 ms. The noise level shows that a CD change of amplitude $\sim 50 \times 10^{-6}$ absorbance units is detectable at 300 μs

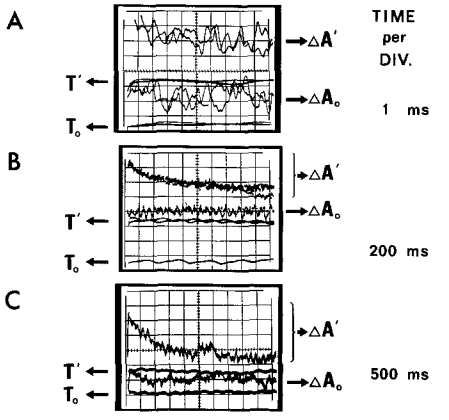

Fig. 1. Temperature-Jump Circular Dichroism observations on the alcohol dehydrogenase-auramine O complex, showing the different relaxation behaviour of circular dichroism (ΔA) and transmission (T). Subscript (o) and superscript (') refer to values before and after the temperature jump respectively. ΔA_o = -260×10^{-6} units, T_o = 17.9%.

Vertical scales: circular dichroism A, 40; B, 40; C, 20 x 10^{-6} units/division; transmission A, B and C 0.1% per division. Horizontal scales: as indicated in Time per division. For other experimental details, see text.

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time constant. Parallel measurements on a comparable solution of AuO alone show no relaxation effects.

The full magnitude of the faster CD effect (Fig 1A) is not fully expressed in Fig 1B due to the longer time constant of the latter measurement. Comparing Figs 1B & 1C (at 10 and 30 ms time constant respectively) the perturbation is seen to be proportional to discharge energy, as expected. The smallness of the transmission transient (0.3%, equivalent to an absorbance change of 0.007) is due to the observing wavelength being close to an isosbestic point for this system (λ = 433 nm) and the finite bandwidth ($\sim$10 nm) of the light used in the T-jump measurement. Any absorbance change in Fig 1B must be less than the noise level (due to arc ripple) which is equivalent to an absorbance of $\pm$ .0002.

From Fig 1A it may be seen that the magnitude of the initial CD transient is +200 x 10^{-6} units, i.e. a large fraction of the equilibrium value at 15°C. Since the T-jump causes only small changes in equilibrium concentrations, this

cannot be accounted for solely by a dissociation process, but requires the presence of two complexes with different properties and most likely of opposite chirality.

The existence of two distinct enzyme-ligand complexes is further substantiated by the fact that there are two relaxation effects in the transient CD, and these are large and of opposite sign. This requires a 2-step mechanism of the form

$$E + AuO \underset{k_b}{\overset{k_f}{\rightleftharpoons}} E.AuO' \rightleftharpoons E.AuO$$

where E.AuO' and E.AuO represent true physical complexes, and E represents the enzyme LADH. These must differ substanially in CD properties, but are effectively identical in absorbance, consistent with their being two distinct conformational states of the enzyme-ligand complex.

The first step in this mechanism given by rate constants k_f and k_b could itself be a two step process analogous to "outer-sphere" complex formation (15) formally involving preliminary formation of an encounter complex E,AuO. This would produce no spectroscopic change as such, but would contribute to the value of τ_1. This possibility was investigated further using the commercial Messanlagen T-jump system to monitor TX changes. An approximate relaxation time for the fast step could be obtained. A solution containing 50 μN enzyme and 40 μN AuO ($\sim$27.5 μN complex) was investigated over the wavelength range 425-450 nm, 20-30 kV discharge and pH range 7-9, giving a value $\tau_1 = 250 \pm 50$ μs.

A full mechanism may be written:

$$E + AuO \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} E,AuO \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} E.AuO' \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} E.AuO$$

given that τ_1 and τ_2 are separated by a factor of greater than 10, and the assumption that E,AuO is negligible in concentration, we may write

(i) $\tau_1^{-1} = k_f [\bar{E} + \overline{AuO}] + k_b$, where $k_f = \frac{k_{12}}{k_{21}} \cdot k_{23}$,

$k_b = k_{32}$, and $\bar{E}$ and $\overline{AuO}$ are equilibrium concentrations of the free components; $\frac{k_{12}}{k_{21}} = K_E$, the stability constant for the encounter complex;

(ii) $\tau_2^{-1} = k_{34} + k_{43}$

The overall equilibrium constant $K_{Eq} = K_D^{-1} = \frac{k_f}{k_b} \cdot \frac{k_{34}}{k_{43}} = 10^5$

Since from the CD result, significant amounts of both complexes are involved, $\frac{k_{34}}{k_{43}}$ must be of the order of unity. Thus :

$\frac{k_f}{k_b} \sim 10^5$; $k_f = 10^8$ $lmole^{-1}s^{-1}$ and $k_b = 10^3 s^{-1}$ from the expression for τ_1. Since $\frac{k_{12}}{k_{21}} \sim 1$, k_{23} must be of the order of $10^8 s^{-1}$.

The ability to observe the additional relaxation effect (τ_2) in CD indicates (i) the mechanism involves more than the steps given by k_f and k_b, (ii) a second molecular complex is present in the equilibrium mixture and (iii) the two complexes differ substantially in their symmetry properties.

As with other transient measurements, the spectroscopic sensitivity achievable at a given time constant depends upon light source power at the photodetector. In these measurements a power of $\sim$ 100 μwatts was readily achieved, and measurements at lower wavelengths in the visible and UV range are currently being pursued.

The technique of temperature jump circular dichroism allows relaxation processes to be studied by the simultaneous observation of two optical parameters which monitor different properties of the reacting system. The method yields additional information on the complexity of reacting systems, provides a means of following rapid conformational relaxation processes which are uniquely sensitive in circular dichroism, and allows the characterisation of reacting molecular species in terms of their distinctive chiral as well as absorption properties. Temperature jump circular dichroism is particularly suited to the study of the rates of conformation change in enzymic systems,

which because of their characteristic kinetic properties show relaxation processes in the time range of 1s to 100 μs.

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