STOPPED-FLOW CIRCULAR DICHROISM: A RAPID KINETIC STUDY OF THE BINDING OF A SULPHONAMIDE DRUG TO BOVINE CARBONIC ANHYDRASE

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The technique of Stopped-Flow Circular Dichroism allows the simultaneous monitoring of chiroptical and absorbance transients at millisecond time resolution. In the binding of a chromophoric sulphonamide to Bovine Carbonic Anhydrase, the rapid kinetics of the induced circular dichroism and difference spectra proceed in parallel with bimolecular rate constant $k_1 \approx 5 \times 10^6$ M^{-1} sec^{-1} and apparent half reaction time of 8.7 msec for 24 μM reactants. A single classical binding process is indicated by both optical parameters.

INTRODUCTION

The technique of Stopped-Flow Circular Dichroism (SFCD) for simultaneous observation of CD and absorbance transient processes has been described previously (1,2). CD is a conformationally sensitive optical property exhibited by all systems with optical asymmetry, and has been extensively used in studying conformational properties of biological molecules (3). Rapid kinetic observations of CD have hitherto been precluded by instrumental limitations, although the desirability of such direct observations has been appreciated for studies of allosteric and related systems (4). The application of SFCD to the study of a ligand binding process allows the observation of the time course of a chiroptical property specifically reflecting the conformational relationship of ligand with its binding site. This additional use of a symmetry-related optical parameter permits a distinction to be drawn between the

binding process as a single step, "lock and key" mechanism, or as a series of sequential inter-related kinetic and conformational steps, as implied by a ligand-induced conformational change.

This first illustrative study reports the binding to bovine carbonic anhydrase (E.C.4.2.1.1) of 5-(p-sulphamyl phenylazo)phenol (SPAP), an analogue of the antimicrobial drug Lutazol. The kinetics of the induced CD and difference spectra have been followed by SFCD at 405 nm. The binding of sulphonamide inhibitors to carbonic anhydrase has previously been monitored for a variety of mammalian isozymes with equilibrium CD and absorbance studies (5) and for the human isozymes B and C with rapid kinetic measurements of protein or ligand fluorescence (6,7). This report establishes the feasibility of millisecond CD observations for monitoring rapid transient optical processes in enzyme-ligand interactions.

EXPERIMENTAL

SFCD measurements were performed with the system described (1,2) using a modified mixer, operated at 7 bar air pressure, and with ratioing electronics in both CD and transmission channels which will be described elsewhere. Observations were made at 405 nm, using a high pressure mercury arc and interference filter, half-bandwidth 10 nm. Multiple shots of the transient and plateau signals were recorded on a Tektronix 564B storage oscilloscope. Bovine carbonic anhydrase was from Sigma, Lot 32C-8150; concentrations of solutions dialysed against tris hydrochloride buffer, pH 7.5, 25 mM were determined from $\varepsilon 280 = 5.70 \times 10^4\ M^{-1}cm^{-1}$ (5,8). The azosulphonamide was the gift of Dr. R. King; solutions were made by weight at alkaline pH and adjusted with dilute HCl. Acetazolamide (Diamox) was from Lederle Laboratories, American Cyanamid Corp. The CD channel was calibrated with 0.02M bornane dione (twice recrystallised from aqueous ethanol). Equilibrium CD and absorption spectra were recorded on the Jouan Dichrographe and CARY 118 spectrophotometer respectively.

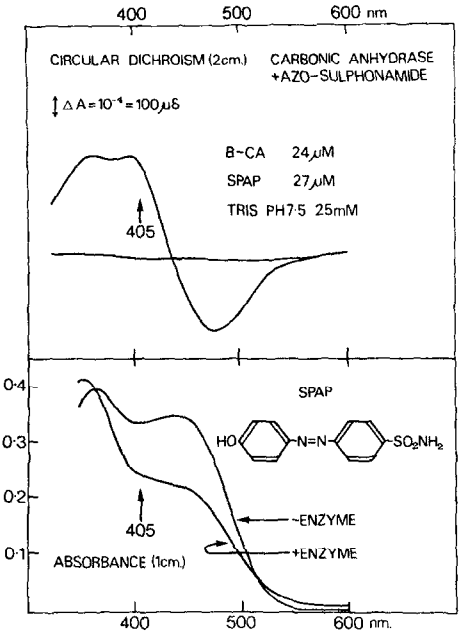

Fig. 1. The binding of the azosulphonamide 5(p-sulphamyl phenylazo) phenol, SPAP, to Bovine Carbonic Anhydrase, CA. Upper spectra, circular dichroism, lower spectra, absorbance.

RESULTS AND DISCUSSION

The binding of SPAP to bovine carbonic anhydrase is characterised by a strong attenuation of the visible absorption spectrum of the bound chromophore, and development of intense CD, Figure 1. The disymmetry ratio $g = \Delta\varepsilon/\varepsilon$ is 0.46×10^{-3} at 405 nm. In rapid kinetics, the binding process is monitored at 405 nm by a decrease in absorbance (i.e. increased transmission, T%) and increased positive CD ($\Delta A = \Delta\varepsilon.c.l.$) The reaction is studied under bimolecular conditions in order to work close to the optimal absorbance (A=0.4 to 0.8, Ref. 2) and at practicable enzyme concentrations. Two such rapid kinetic experiments are shown in Fig. 2. The traces are triggered from the stop signal of the mixing process, monitored by an accelerometer on the driving syringe. The experimental dead-time (9), defined as the average life time of the observed mixture is 0.75 msec. (In the longitudinal observation system, path length 2.1 cm, this

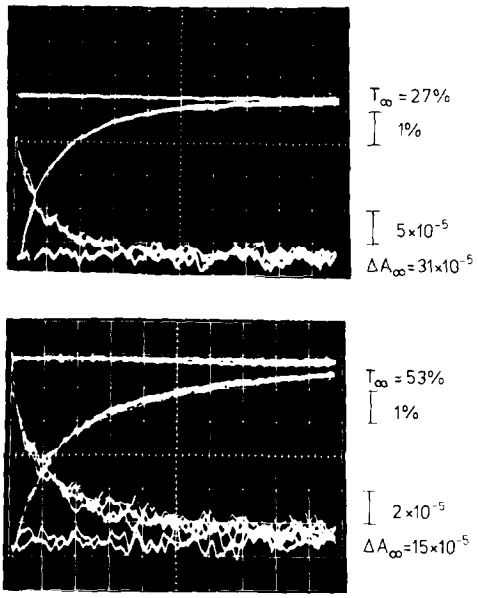

Fig. 2. Stopped Flow Circular Dichroism traces: the kinetics of binding of SPAP to CA, observed at λ = 405 nm. Upper trace transmission, T, lower trace circular dichroism, ΔA; transients and plateau ($t = \infty$) values are shown. Syringe concentrations: Upper photo, CA 48 μM, SPAP 54 μM; Lower photo CA 24 μM, SPAP 27 μM. Time constants, transmission and CD 3 ms. Time base 20 ms/div.

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introduce a loss of total amplitude of ~5% for an exponential lifetime of 8 msec).

The transients recorded are the results of multiple shots taken over a period of 1 minute; plateau values are then recorded. For calculation from the photograph, points are taken every 0.2 divs; CD and absorbance values are analysed by a least-squares procedure for the optimal fit to a given rate equation in terms of both rate constant and absolute plateau values. The longest instrumental time constant (3 msec) is appreciably shorter than the observed transients, and no deconvolution was attempted.

The results clearly show that CD and absorbance signals corresponding to the full amplitude difference between free and bound equilibrium states are observed as single transients. Also, the time course of the two signals is apparently identical within experimental limits. From Fig. 2A and 2B, half reaction is complete in 9 and 18 msec respectively. Analysis

of 8 traces in terms of the second order rate-equation gave k_1 values 5×10^6, and 4×10^6 M^{-1} sec^{-1} at 24 and 12 μM reactants respectively. Displacement of SPAP from the complex with a 20 x molar excess of acetazolamide showed parallel CD and absorbance changes with first order $t_{\frac{1}{2}} > 30$ sec, $k_1 < 0.033$ sec^{-1}, calculated Kequil $> 1.5 \times 10^8$ M^{-1}.

The value of $k_1 = 5 \times 10^6$ M^{-1} sec^{-1} is significantly less than that for a diffusion-controlled process, $k_D \sim 10^9$ M^{-1} sec^{-1} (10). This phenomenon has also been observed for the quenching of protein fluorescence in a similar system (6), and interpreted as involving an initial complex analogous to "outer-sphere" complex formation (11). The observed value is close to those of 1.13×10^7 and 6.8×10^6 M^{-1} sec^{-1} for the salicylyl derivative (Lutazol) with the human isozymes B and C, and for which Kequil $> 10^8$ M^{-1}.

The chiral state of the bound chromophore is determined by a variety of interactions with neighbouring groups in the protein; it exhibits different CD with other isozymes (12). A similar result is found for equilibria of a sulphamylphenyl-azonaphthalene derivative (5). It is clear from the kinetic results that this chiral state is reached without a conformational change in the enzyme following the initial liganding. This process thus corresponds at this time scale to the "lock and key" mechanism of classical enzymology.

These measurements have therefore demonstrated the feasibility of following rapid kinetic changes by CD. A fuller kinetic study of other ligands with additional isozymes has been made and will be reported elsewhere (12).

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