A New, General Method for the Determination of Binding Constants: Photoacoustic Titration of Dansylamide with Carbonic Anhydrase

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We have developed a new method utilizing the partitioning of energy between radiative and non-radiative pathways following photostimulation for the determination of binding constants in biochemical systems; application of this photoacoustic calorimetry technique to the study of the inhibition of carbonic anhydrase by 1-(dimethylamino)-5-naphthalenesulphonamide yields K_{11} = 1.7 (±0.4) × 10^6 mol^{-1} dm^3.

Photoacoustic calorimetry (PAC) has been applied by Peters,[1] Braslavsky,[2] Griller,[3] and others[4] to the determination of thermodynamic parameters and/or dynamics in a number of chemical systems, including the photodissociation of carbon monoxide from myoglobin,[1b] the displacement of heptane from $Cr(CO)_5$heptane by pyridine,[1c] and the measurement of bond dissociation energies.[3] We wish to report the first application of PAC to the determination of the affinity of a receptor for a ligand.

Carbonic anhydrase (bovine erythrocyte, mixture of isozy-

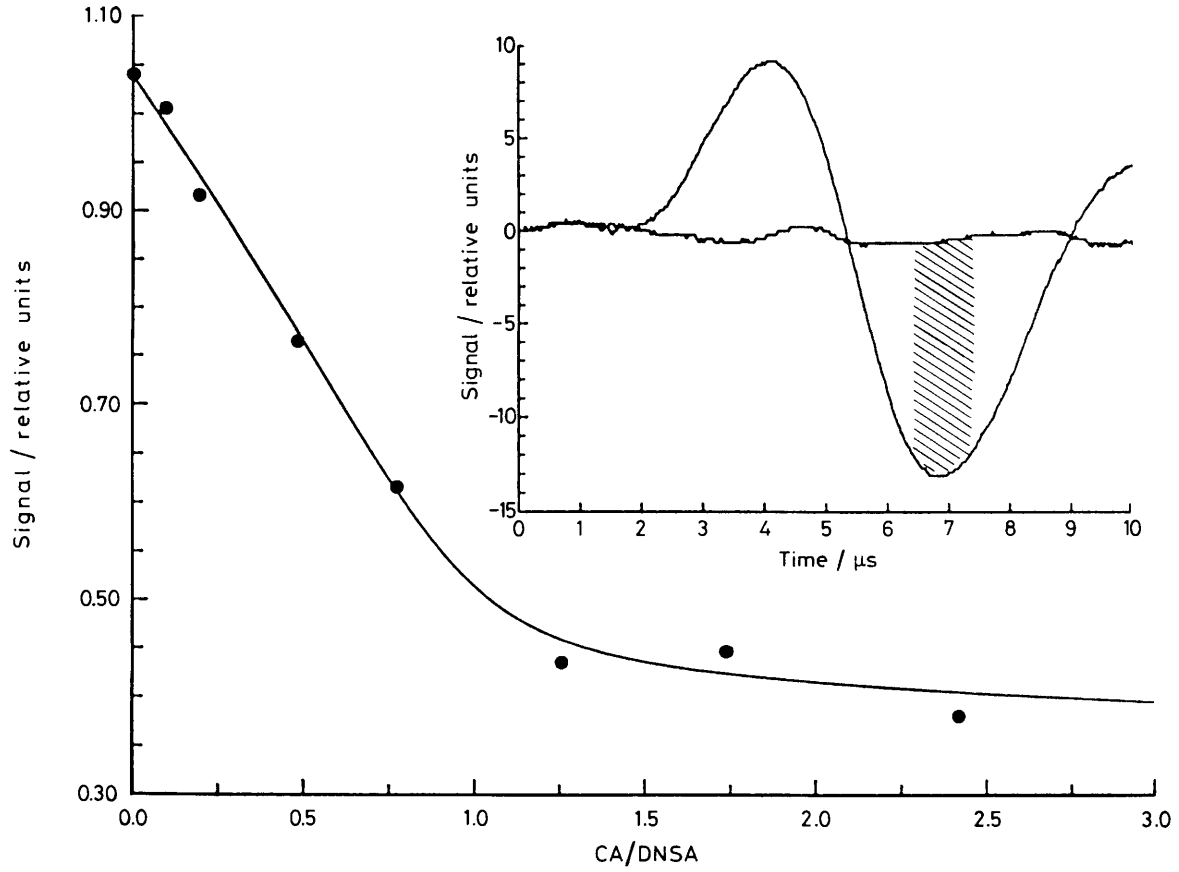

Figure 1. A typical photoacoustic titration curve for dansylamide–carbonic anhydrase. Circles represent experimental data; the curve is the calculated best fit, corresponding to $K_{11} = 1.9 \times 10^6$ mol^{-1} dm^3. (DNSA = dansylamide; CA = carbonic anhydrase; $[DNSA]_0 = 2.04 \times 10^{-5}$ M; $\lambda_{exc} = 337.1$ nm; pulse energy ~20 μJ). Inset: A typical photoacoustic wave for dansylamide in water; the experimental baseline (signal for aqueous buffer alone) and the integration limits (used to obtain the signal amplitude) are also indicated.

mes) interacts with 1-(dimethylamino)-5-naphthalenesulphonamide (dansylamide) to afford a 1:1 complex.[5] The change in fluorescence of dansylamide observed upon its binding to carbonic anhydrase is the basis of an existing assay for the determination of binding constants of non-fluorescent inhibitors to this protein.[6] Herein we describe a new, general method which utilizes the difference in quantum yields for non-radiative relaxation exhibited by free and bound dansylamide. This difference in thermal energy deposition to the solution is used to monitor the extent of complex formation as a known concentration of inhibitor is titrated with protein.† Dansylamide, bound to carbonic anhydrase or free in solution, absorbs photons at 337.1 nm (N_2 laser); the free ligand rapidly returns most of the absorbed energy to the solution as heat (Φ_f is small;[7] $f_h = 0.978 \pm 0.006$),[8]‡ whereas the enzyme-bound molecule returns a smaller fraction of the excitation energy as heat (f_h ~0.3)‡ and emits the rest as fluorescence ($\Phi_f = 0.84$).[5] Any non-radiative relaxation will produce a local temperature increase, which gives rise to an adiabatic, isobaric expansion, which in turn creates a pressure wave (acoustic wave) in solution. This pressure wave passes through the solution and is readily transmitted through the quartz wall of a standard cuvette and into a piezoelectric based transducer that is clamped to the outside of the cell. An example of a detected photoacoustic wave for dansylamide in aqueous buffer is shown in the inset of Figure 1.

A PAC experiment to determine a binding constant is executed in the following manner: Degassed dansylamide in 0.3% methanol–20 mM aqueous KH_2PO_4 buffer (pH 7.4) is placed in the sample cuvette, at room temperature, and photoacoustic waves generated from this sample upon irradiation by a short, low energy laser pulse (≤20 μJ) are digitized, collected, and averaged for 35 laser shots.§ The equilibrium concentrations of inhibitor, protein, and their complex are then perturbed by increasing the protein concentration and a new set of data collected. Over the course of this titraton of a fixed concentration of dansylamide, we observe a decrease in the amplitude of the photoacoustic signal that scales with the amount of carbonic anhydrase added. The data are analysed by integration of the first negative excursion of the photoacoustic waves (shaded area of Figure 1 inset) for each ratio of [carbonic anhydrase]-to-[dansylamide] and are then normalized to laser energy, which is also measured for each pulse during the experiment.[9]

The set of integrated, normalized photoacoustic signals from the titration are fitted by a non-linear least-squares routine to an expression for a 1:1 binding interaction. Typical data from one experiment are shown in Figure 1. The binding constant, obtained as the average from five separate experiments (± one standard deviation) is 1.7 (±0.4) × 10^6

† We also can and have obtained satisfactory data from the titration of carbonic anhydrase with dansylamide, an assay requiring less protein.

‡ The variable f_h is the fraction of absorbed energy released as heat.

§ No waveform shifts were observed upon titration, indicating that heat deposition is always occurring in the same time frame.

mol^{-1} dm^3, in excellent agreement with a binding constant of 2.1 ($\pm$0.5) $\times$ 10^6 mol^{-1} dm^3 obtained from non-linear fitting of data obtained by the fluorescence binding assay.[10] These values are in accord with previous measurements of this binding constant [4.2 ($\pm$0.2) $\times$ 10^6 mol^{-1} dm^3;[5] 5.0 $\times$ 10^5 mol^{-1} dm^3].[6]

The PAC binding assay is both experimentally straightforward and versatile. Any system comprised of bound and free populations for which the non-radiative transitions following photostimulation are different for these species can in principle be examined by PAC. Changes in quantum yields for fluorescent and non-radiative processes or in time constants for energy deposition are two important parameters that the PAC technique can exploit, as well as changes in extinction coefficients for the species being studied. The method has obvious application where background fluorescence or light scattering interferes with measurements of fluorescence of the group of interest, or where luminescence is not easily monitored. We are now pursuing other systems for which PAC offers a unique solution to problems in molecular recognition.

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