FARADAY

Kinetics and equilibrium binding of the dyes TNS and RH421 to ribulose 1,5-bisphosphate carboxylase/oxygenase (RUBISCO)

Joachim Frank,[a] Josef F. Holzwarth,[a] Arie van Hoek,[b] Antonie J. W. G. Visser[b] and Joachim Vater[c]

[a] *Fritz-Haber-Institut, Max-Planck-Society, Faradayweg 4-6, D-14195 Berlin, Germany*

[b] *Department of Biochemistry, Agricultural University, Dreijenlaan 3, 6703 HA Wageningen, The Netherlands*

[c] *Max-Volmer Institut für Biophysikalische Chemie und Biochemie, Technische Universität Berlin, Franklinstr. 29, D-10587 Berlin, Germany*

2-(*p*-Toluidino)naphthalene-6-sulfonate (TNS) binds in a reversible bimolecular reaction non-covalently to RUBISCO, the water-soluble enzyme for carbon dioxide fixation. TNS does not change the substrate activity at the active site of RUBISCO. Rate constants k_+ and k_- for the association and dissociation were measured as $(1.2 \pm 0.2) \times 10^7$ dm^3 mol^{-1} s^{-1} and 1020 ± 300 s^{-1}, respectively. The binding of the steryl dye *N*-(4-sulfobutyl)-4-{4-[*p*-(dipentylamino)phenyl]butadienyl}pyridinium inner salt (RH421) to RUBISCO is a diffusion-controlled reversible bimolecular reaction with an association rate constant k_+ of $(7 \pm 0.6) \times 10^9$ dm^3 mol^{-1} s^{-1} and a dissociation rate constant k_- of $(1.8 \pm 0.2) \times 10^4$ s^{-1}. The dissociation constants K_d for the binding of TNS and RH421 to RUBISCO were calculated from the kinetically determined rate constants. These data are in good agreement with the dissociation constants measured by equilibrium techniques. Upon binding to RUBISCO the fluorescence of TNS and RH421 is more intense and blue shifted. The fluorescence lifetimes of TNS and RH421 are longer compared to those of both dyes dissolved in aqueous solution. The fluorescence anisotropy correlation time of 200 ns for TNS bound to RUBISCO corresponds to a rigidly bound dye which rotates with the same rotational correlation time as the whole protein. Both dyes can be used as probes sensitive to their molecular environment. In further experiments they were applied to the detection of ligand binding events at the active site of RUBISCO as will be described in a forthcoming publication.

The fluorescent dye TNS [Fig. 1(A)] was introduced by McClure and Edelman as a probe for the detection of conformational changes of proteins.[1–3] TNS is only weakly fluorescent in polar solvents but upon binding to hydrophobic regions of proteins or when dissolved in non-polar solvents the fluorescence of the dye strongly increases and the maximum of the fluorescence spectrum is shifted to shorter wavelengths. TNS was used by several authors as a probe for the detection of conformational changes and ligand binding events of enzymes.[3–5] It reacts as an environmentally sensitive probe which detects polarity and viscosity changes in its surroundings.[6,7] Previously it was shown by Visser *et al.* that the steryl dye RH421 [Fig. 1(B)] binds directly to proteins.[8] The fluorescence properties of RH421[9] bound to RUBISCO are comparable with those of TNS.[1–3] Both dyes are sensitive to their environment and show a blue shift and an increase of fluorescence on binding to RUBISCO. It was demonstrated that TNS does not change the carboxylase activity of RUBISCO in the concentration range used in this investigation.[10a,b] To clarify the binding properties of both dyes for kinetic studies of ligand binding to RUBISCO we investigated the interaction of the dyes with the water-soluble enzyme alone using fluorescence titration and fluorescence lifetime measurements as well as fast reaction techniques. The hexadecameric RUBISCO of higher plants has a molecular weight of 550 000 consisting of eight large catalytic (L) and eight small regulatory (S) subunits.[11] It is the key enzyme of the carbon reductive pathway (Calvin cycle) and of photorespiration. RUBISCO catalyses both the carboxylation and the oxygenation of the substrate D-ribulose 1,5-bisphosphate (RUBP) and shows activation by carbon dioxide and magnesium ions.[12,13]

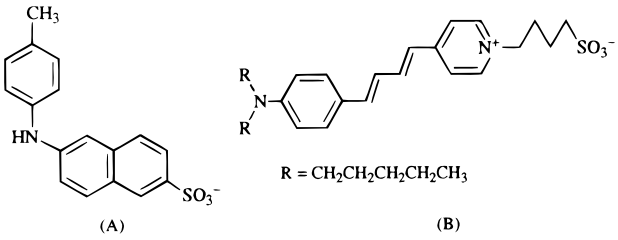

Fig. 1 Structure of TNS (A) and RH421 (B)

Experimental

RUBISCO from spinach was purified close to homogeneity as described by Vater *et al.* and stored after addition of 10% w/v glycerol at −20 °C.[10b] The mean value of the specific activities of the enzyme preparations was between 1 and 1.5 units mg^{-1}. RUBISCO from spinach was dialysed prior to use against 20 mM TRIS-HCl buffer of pH 8.0 (1 mM dithioerythritol, 0.1 mM EDTA) at 4 °C.

RUBISCO from *Rhodospirillum rubrum* was a kind gift from G. H. Lorimer (DuPont, USA). After long-term storage at −20 °C the bacterial enzyme was treated with 50 mM dithioerythritol and 5 mM EDTA according to Tabita and McFadden and dialysed prior to use against 20 mM TRIS-HCl buffer of pH 8.0 (1 mM dithioerythritol, 0.1 mM EDTA) at 4 °C.[14,15]

The dyes TNS and RH421 were purchased from Serva (Germany) and Molecular Probes (USA), respectively.

The binding of the fluorescent probe TNS to RUBISCO from spinach and *Rhodospirillum rubrum* was recorded with a fluorescence spectrometer (Shimadzu RF 5000) at excitation/

emission wavelengths of 366/427 nm at constant protein concentrations between 0.1 (0.187 μM) and 0.3 mg ml^{-1} (0.56 μM) for RUBISCO from spinach and 0.53 mg ml^{-1} (0.47 μM) for RUBISCO from *Rhodospirillum rubrum* at pH 8.0 and 25 °C. A TNS stock solution dissolved in 20 mM TRIS-HCl buffer of pH 8.0 (1 mM dithioerythritol, 0.1 mM EDTA) was mixed with an enzyme solution yielding dye concentrations between 5 and 250 μM. The measured fluorescence yield at 427 nm was plotted as a function of the TNS concentration and fitted by a least-squares non-linear regression program to a Michaelis–Menten like equation for the fluorescence (F) as a function of the dye (D) concentration: $F = F_{max}[D]/(K_d + [D])$ (ENZFITTER, least-squares regression program purchased from Biosoft, Cambridge, UK). For the estimation of the number n of the binding sites for TNS and the apparent dissociation constant K_d the mass-law equation was used. The amount of bound dye was calculated by the following formula:

$$[D]_{bound} = (B/2) - \{(B/2)^2 - n[E]_{total}[D]_{total}\}^{1/2},$$
$$B = [D]_{total} + [E]_{total} + K_d \quad (I)$$

The experimental data for the fluorescence as a function of the dye concentration at a constant enzyme concentration were fitted with the aid of least-squares non-linear regression to the above equation by introducing a proportionality constant between bound dye and detected fluorescence ($F = C[D]_{bound}$).

The stoichiometry of the binding of TNS to RUBISCO from spinach was investigated by an ultrafiltration technique using an Amicon system. The amount of bound and free TNS in solutions containing a constant RUBISCO concentration of 0.3 mg ml^{-1} (0.561 μM) was calculated from the difference in the TNS concentration measured by the absorption at 366 nm between the upper macromolecular phase and the lower free TNS phase, after a rapid separation of the macromolecule from the unbound TNS by centrifugation of the solution through a X100 membrane filter with a cut-off of 100 000 u (Amicon). The ratio of bound TNS/RUBISCO was then plotted as a function of the free TNS concentration and was extrapolated to an infinite free TNS concentration to determine the number of binding sites of RUBISCO from spinach for TNS.

Fluorescence titrations of RUBISCO from spinach with RH421 (excess of dye over protein) were performed to determine the binding constant K at excitation/emission wavelengths of 550/648 nm and a constant protein concentration of 0.1 mg ml^{-1} (0.187 μM) at pH 8.0 and 25 °C. A 1.3 mM stock solution of RH421 dissolved in methanol was mixed with an enzyme solution resulting in dye concentrations between 0.1 and 10 μM. The product nK was determined in a second titration of a dye solution with protein in excess according to Frank *et al.*[9] The results of both titrations yielded the number of binding sites n and the binding constant K for the binding of RH421 to RUBISCO from spinach.

Emission spectra of TNS bound to RUBISCO were recorded between 300 and 550 nm (excitation wavelength 280 nm) as a function of dye concentration at a constant protein concentration at pH 8.0 and 25 °C.

Fluorescence quantum yields φ of the free dye in solution and of the dye bound to RUBISCO were estimated by the use of quinine sulfate as a standard as described by McClure and Edelman.[1] At a constant TNS concentration of 10 μM the dye was titrated with an excess of RUBISCO. After each addition of the protein, emission spectra were recorded, using an excitation wavelength of 366 nm. The area of the fluorescence emission spectra was plotted as a function of the RUBISCO concentration. The fluorescence showed saturation characteristics at an excess of RUBISCO. The maximum area of the fluorescence band measured under these conditions was extrapolated to infinite RUBISCO concentration and compared with the area of the fluorescence band of a 10 μM quinine sulfate standard to determine the fluorescence quantum yield φ of TNS interacting with RUBISCO.

Polarized fluorescence decay of TNS in the presence and absence of RUBISCO was measured using a picosecond laser system in combination with time-correlated single photon counting as described in detail elsewhere.[16–18] The excitation wavelength was 340 nm and the fluorescence was detected at either 434 or 489 nm using a combination of a 434/489 nm interference filter (Schott, Mainz, Germany) and a WG 360 nm (Schott) cut-off filter. The instrumental response function, corresponding to the laser pulse convoluted with the detection response, was determined by measuring the fluorescence decay of xanthione in n-hexane (spectroscopic grade, Merck) resulting in a lifetime of 25 ps.[19] Polarized fluorescence intensity components $I_{\parallel}(t)$ and $I_{\perp}(t)$ were registered.

From these data the total fluorescence decay $S(t) = I_{\parallel}(t) + 2I_{\perp}(t)$ and the fluorescence anisotropy decay $r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)]$ were obtained. The total fluorescence decay $S(t)$ was fitted using the global analysis program described by Beechem *et al.*[20] (Globals Unlimited TM, Urbana, IL, USA) to the following equation: $S(t) = \Sigma\alpha_i \exp(-t/\tau_i)$; α_i is the pre-exponential factor belonging to the lifetime component τ_i with a minimum number of lifetimes to obtain an acceptable fit to the data ($\chi^2 \approx 1.0$–1.5 and randomly scattered weighted residuals between experimental and fitted decay curves). It was found that the fluorescence decay of free TNS in aqueous solution can be characterized by a predominantly present component of 17 ps. The fluorescence decay of protein-bound TNS is much longer and required two or three components (depending on the conditions) to have an acceptable fit to the experimental data. The first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ average lifetimes of the dye fluorescence were calculated from the fitted values of α_i and τ_i according to the following equations: $\langle\tau_1\rangle = \Sigma\alpha_i\tau_i/\Sigma\alpha_i$ and $\langle\tau_2\rangle = \Sigma\alpha_i\tau_i^2/\Sigma\alpha_i\tau_i$. First-order average lifetimes have been used because they are directly comparable with the fluorescence intensity from steady-state fluorescence experiments.[21] The second-order lifetime corresponds to the true fluorescence lifetime. The fluorescence anisotropy decay $r(t)$ was fitted to a single exponential equation by using the global analysis program: $r(t) = \Sigma\beta_i \exp(-t/\phi_i)$ where β_i is the pre-exponential factor belonging to the correlation time ϕ_i.

Isothermal differential titration calorimetry (ITC) experiments of the TNS binding to RUBISCO were performed using the OMEGA high-sensitive microcalorimeter manufactured by MicroCal Inc. (Northampton, MA, USA). A detailed description of the design and operation of this instrument has been provided previously.[22] The instrument was calibrated by measuring the area under a known electric test pulse and comparing it with the heat released. For measurements of the heat production accompanying the binding of TNS to RUBISCO the enzyme was loaded into the sample cell of the calorimeter (volume = 1.3592 ml) and the reference cell was filled with water containing 0.05% sodium azide. The value C, defined as the product of the binding constant K and the concentration of binding sites was between 0.1 and 1; these are not the best conditions for measurements but they could not be changed because of RUBISCO and TNS concentration restrictions. TNS in the same buffer as RUBISCO was filled into a 250 μl syringe at a concentration of 6.57 mM. The system was allowed to equilibrate and a stable baseline was recorded before initiating an automated titration. A typical experiment involved 25 injections of 10 μl aliquots of TNS at intervals of 3 min. Throughout the titration the cell was stirred continuously at 400 rev min^{-1}. The presented results correspond to experimental traces of heat evolved *vs.* time (injection number). The obtained data were evaluated to determine the binding stoichiometry n, binding constant K

and the associated enthalpy change $\Delta_b H$ by non-linear least-squares analysis in terms of the following equation:

$$Q_t = nM_t\,\Delta_b HV\{(1 + X_t/nM_t + 1/nKM_t) - [(1 + X_t/nM_t + 1/nKM_t)^2 - 4X_t/nM_t]^{1/2}\}/2 \quad \text{(II)}$$

where M_t is the total RUBISCO concentration and V is the cell volume. Using this equation the total heat Q_t was then fitted *via* a non-linear least-squares minimization method to the total titrant concentration X_t. The expression for the measured difference $\Delta Q_i = Q_i - Q_{i-1}$ is given by:[22]

$$\Delta Q_i = Q_i + [(dV_i/2V)(Q_i + Q_{i+1})/2] - Q_{i-1} \quad \text{(III)}$$

where dV_i is the volume of titrant added to the solution in the calorimeter cell.

The iodine laser temperature jump technique (ILTJ)[23] was applied to equilibrium mixtures of enzyme and dye. The relaxation of the concentrations of bound and unbound dye to a new equilibrium state was followed by recording the change of fluorescence intensity after a fast (2.3 μs) temperature jump of 1 K in the time range from 1 μs to 1 s.[23] The time constants of the ILTJ relaxations for the interaction of TNS with RUBISCO were measured as a function of the dye concentration at enzyme concentrations between 0.1 and 0.5 mg ml^{-1} and as a function of the enzyme concentration at a dye concentration of 10 μM at pH 8 and 25 °C in 20 mM TRIS buffer (1 mM dithioerythritol and 0.1 mM EDTA). The time constants of the ILTJ relaxations for the interaction of RH421 with RUBISCO from spinach were measured as a function of the dye concentration keeping the enzyme concentration constant (0.25 mg ml^{-1}) at pH 8 and 25 °C in 20 mM TRIS buffer (1 mM dithioerythritol and 0.1 mM EDTA). The relaxation times τ and the corresponding amplitudes A were estimated as parameters of a least-squares fit of the relaxation curves utilizing a Marquardt algorithm. For pseudo-first-order conditions ([dye] ≫ [enzyme] or [enzyme] ≫ [dye]) the reciprocal relaxation time $1/\tau$ was calculated and plotted as a function of dye concentrations at constant enzyme concentration or as a function of enzyme concentrations at constant dye concentration. In the case of a bimolecular reaction between dye and enzyme $1/\tau$ is a linear function of the dye concentration at constant enzyme concentration or *vice versa*.

Results

Fluorescence titrations of RUBISCO from spinach with TNS showed an increase in fluorescence intensity at 427 nm with increasing TNS concentration due to dye binding (Fig. 2). The carboxylase activity of RUBISCO was not changed by TNS concentrations below 0.2 mM.[10a,b] The fluorescence maximum of bound TNS was observed at an emission wavelength of 427 nm (excitation wavelength 366 nm). Free TNS in aqueous solution has an emission maximum at 500 nm.[1] TNS bound to RUBISCO also quenches the protein fluorescence (Fig. 3). The apparent dissociation constant K_d for the binding of TNS to RUBISCO and the number of binding sites n for the dye under various activation conditions of the enzyme were calculated from the concentration dependence of the fluorescence increase at 427 nm according to eqn. (I). Values of K_d of 98 ± 10 μM and 39 ± 17 μM were determined for the binding of TNS to the inactive forms of RUBISCO from spinach and the bacterial enzyme from *Rhodospirillum rubrum*, respectively. Under these conditions the number of binding sites $n = 37 \pm 8$ of RUBISCO from spinach for TNS was estimated. A similar value of $n = 40 \pm 10$ was determined by ultrafiltration experiments. In the activated state of the enzyme a decrease of K_d to 70 μM and an increase of the fluorescence quantum yield from 0.17 to 0.24 was observed without significant change in the number of bound dye molecules. Similar data were measured for the binding of the transition-state analogue 2-CABP to RUBISCO (see Table 1).

The fluorescence lifetime measurements of TNS bound to RUBISCO resulted in much longer average first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ fluorescence lifetimes compared to free dye in aqueous solution (Table 2). In the presence of magnesium ions, the substrate ribulose 1,5-bisphosphate (RUBP) or the transition-state analogue 2-carboxy-D-arabinitol 1,5-bisphosphate (2-CABP) a decrease in the average first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ fluorescence lifetimes of 10 to 15% was observed (Table 2). Fig. 4(A) presents the total fluorescence

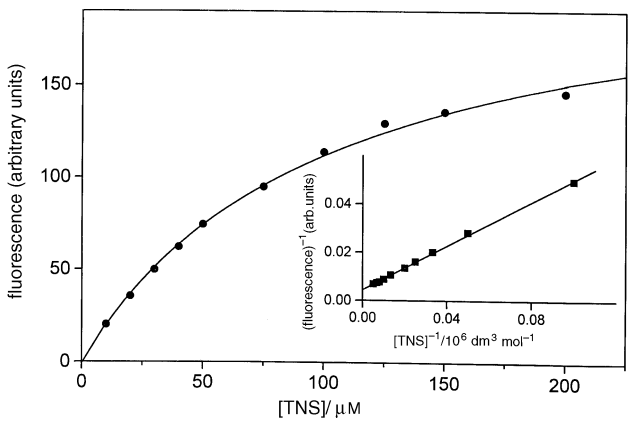

Fig. 2 Determination of the dissociation constant of the spinach RUBISCO–TNS complex (enzyme in the inactive state) obtained from the increase of fluorescence at 427 nm. The insert shows the double reciprocal plot of the titration data. Excitation wavelength 366 nm. RUBISCO concentration = 0.187 μM (0.1 mg ml^{-1}).

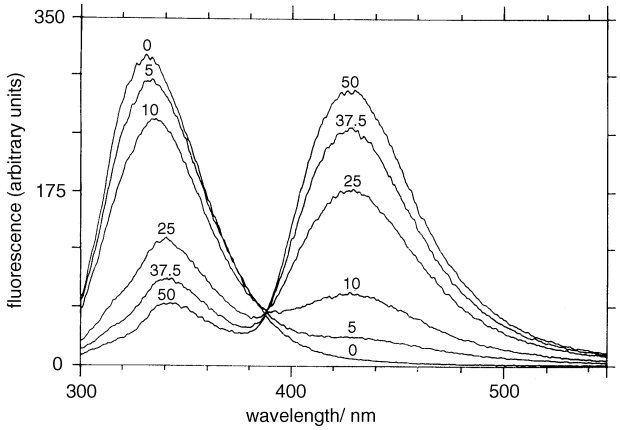

Fig. 3 Emission spectra (excitation wavelength 280 nm) of RUBISCO from *Rhodospirillum rubrum* in the presence of increasing TNS concentrations (0, 5, 10, 25, 37.5 and 50 μM). The decrease of the fluorescence at 340 nm and the increase of the fluorescence intensity at 427 nm is probably due to energy transfer mainly from the tryptophane residues of RUBISCO to the bound dye TNS.

Table 1 Number of binding sites n, apparent dissociation constants K_d and fluorescence quantum yields φ for the binding of TNS to RUBISCO from spinach[a]

RUBISCO	n	$K_d/10^{-6}$ mol dm^{-3}	φ
inactive	37 ± 8	98 ± 10	0.17 ± 0.02
active	51 ± 9	70 ± 12	0.24 ± 0.02
active + 2-CABP	43 ± 14	67 ± 8	0.22 ± 0.03

[a] Inactive state (without Mg^{2+} and HCO_3^-) and active state (in the presence of 10 mM Mg^{2+} and 40 mM HCO_3^-) in 20 mM TRIS-HCl buffer pH 8 (1 mM dithioerythritol and 0.1 mM EDTA) at 25 °C. For free TNS in aqueous solution a quantum yield φ of 8×10^{-4} was estimated by McClure and Edelman.[1]

Table 2 Average first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ fluorescence lifetimes[a]

	$\langle\tau_1\rangle$/ns	$\langle\tau_2\rangle$/ns
TNS (free)	0.019 ± 0.003	0.09 ± 0.013
TNS–RUBISCO	8.8 ± 1.3	11.0 ± 1.6
TNS–RUBISCO + Mg^{2+}	8.7 ± 1.3	10.3 ± 1.6
TNS–RUBISCO + CABP + Mg^{2+}	8.5 ± 1.3	9.9 ± 1.5
TNS/RUBISCO + RUBP	7.9 ± 1.2	9.7 ± 1.5

[a] For 10 μM 'free' TNS in aqueous solution and 10 μM TNS in the presence of 0.3 mg ml^{-1} (0.56 μM) RUBISCO in different activation states at pH 8.0 and 20 °C (fluorescence excitation at 340 nm and detection at 434 nm).

decay curves for TNS in the absence and presence of RUBISCO. It can be clearly seen that the fluorescence decay of TNS in water is ultrafast with a major component of 17 ps and a minor contribution of nanosecond components (the latter contribution is even less when the detection wavelength is at 489 nm which is closer to the maximum of the fluorescence spectrum of TNS in water). The fluorescence decay of RUBISCO-bound TNS is much slower with decay components of 3.5 and 11.5 ns. However, superimposed on the slow fluorescence decay a rapid component arising from unbound TNS is clearly visible. From the fluorescence anisotropy decay curve of TNS in the presence of RUBISCO [Fig. 4(B)] a rotational correlation time ϕ of 200 ns was determined.

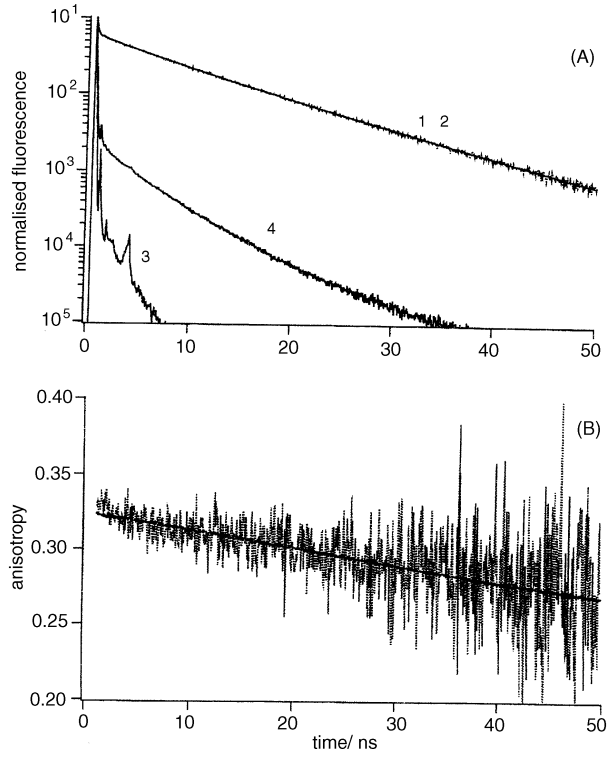

Fig. 4 (A) Fluorescence decays measured at 434 nm by time-correlated single photon counting. Curves 1 and 2: experimental (···) and fitted (——) fluorescence decay for 2.5 μM TNS in the presence of 0.15 mg ml^{-1} (0.28 μM) inactive RUBISCO at pH 8.0 and 20 °C. Curve 3 represents the xanthione fluorescence decay used as instrumental response for deconvolution of the fluorescence decay. Curve 4 is the experimental fluorescence decay of 25 μM TNS in aqueous solution. All curves have been normalized to 10^5. The results of the decay analysis are: $\alpha_1 = 0.888$, $\tau_1 = 17$ ps, $\alpha_2 = 0.021$, $\tau_2 = 0.85$ ns, $\alpha_3 = 0.056$, $\tau_3 = 3.5$ ns, $\alpha_4 = 0.035$, $\tau_4 = 11.5$ ns with $\chi^2 = 1.08$. (B) Experimental (···) and fitted (——) fluorescence anisotropy decay for 2.5 μM TNS in the presence of 0.15 mg ml^{-1} (0.28 μM) inactive RUBISCO at pH 8.0 and 20 °C. The results of the decay analysis are: $\beta = 0.324$ and $\phi = 200$ ns with $\chi^2 = 1.04$. A detailed error analysis (at 67% confidence level) yielded lower and upper bounds for ϕ of 160 and 270 ns, respectively.

Isothermal differential titration calorimetry experiments were performed to test if the binding of TNS to RUBISCO is accompanied by a significant enthalpy change, which is a prerequisite for the ILTJ application.

Fig. 5(A), (B) shows a diagram of the heat in kJ mol^{-1} of injectant and of the heat evolved for each of 25 injections of a concentrated stock solution of TNS into a RUBISCO solution. Values of K, n, and the enthalpy change of binding $\Delta_b H$ could not be determined exactly, since $C = n[\text{RUBISCO}]K$ was between 0.1 and 1 (optimum 0.1–100), so that no clear transition point in the injection/heat dependencies of Fig. 5(B) could be detected. The ITC experiments for the binding of TNS to RUBISCO from spinach allowed us to estimate an exothermic (negative) enthalpy change $\Delta_b H$ of -6.5 ± 1 kJ mol^{-1} [Fig. 5(A), (B)].

The kinetics of the interaction between TNS and RUBISCO were investigated by ILTJ experiments in the time range 1 μs–1 s. Temperature scans of the system TNS/RUBISCO were measured in a fluorescence spectrometer at excitation/emission wavelengths of 366/427 nm between 15 and 30 °C. These experiments showed a change of fluorescence $\Delta F/F$ per °C of 3.8%. Two dye dependent relaxations could be detected (Fig. 6). The first fast relaxation process τ_1 with a relaxation time between 10 and 20 μs corresponds to the protonation/deprotonation reaction of the dye. The slower relaxation τ_2 can be attributed to the dye binding reaction, because the reciprocal relaxation time $1/\tau_2$ is a linear function of the dye concentration at constant enzyme concentration. Similarly, $1/\tau_2$ at constant dye concentration depends linearly on the enzyme concentration as expected for a bimolecular

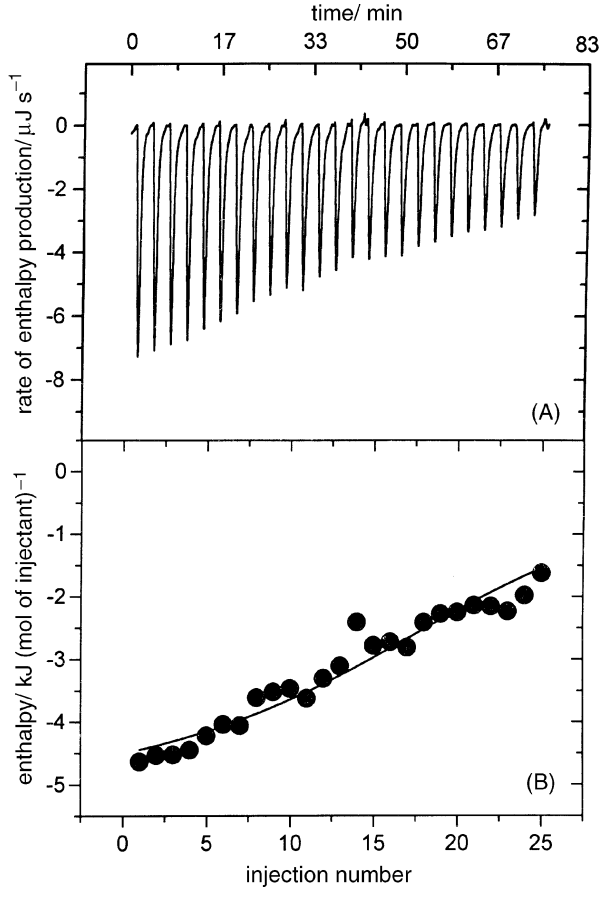

Fig. 5 Diagram of the heat in kJ mol^{-1} per injectant (B) and of the heat enthalpy produced in each (A) of 25 injections of 10 μl of 6.57 mM TNS into a solution of 8.2 μM (4.4 mg ml^{-1}) inactive RUBISCO in 20 mM TRIS-HCl buffer pH 8.0 in the presence of 1 mM dithioerythritol and 0.1 mM EDTA at 22 °C measured by isothermal differential titration calorimetry.

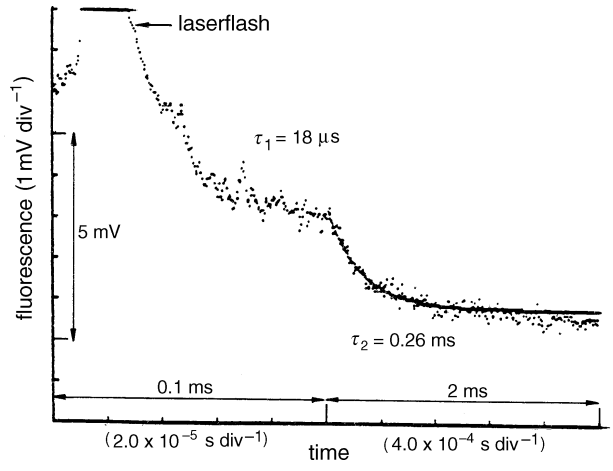

Fig. 6 Detection of the binding of TNS to RUBISCO by the ILTJ technique. Two relaxation signals were observed indicating two fast processes with relaxation times τ_1 and τ_2 at 25 °C in a solution of 100 µM TNS and 0.93 µM (0.5 mg ml^{-1}) inactive RUBISCO from spinach in 20 mM TRIS-HCl buffer pH 8.0 in the presence of 1 mM dithioerythritol and 0.1 mM EDTA; τ_1 was attributed to the protonation/deprotonation equilibrium and τ_2 represents the binding of TNS to RUBISCO.

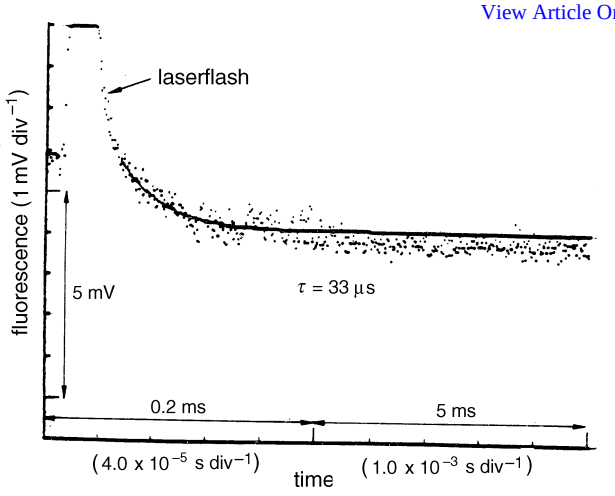

Fig. 8 Detection of the fast binding of RH421 to inactive RUBISCO from spinach by the ILTJ technique. In this case only one relaxation signal was observed which represents a fast process with a relaxation time of 33 µs at 25 °C in a solution of 3.5 µM RH421 and 0.47 µM (0.25 mg ml^{-1}) RUBISCO from spinach in 20 mM TRIS-HCl buffer pH 8.0 in the presence of 1 mM dithioerythritol and 0.1 mM EDTA.

reaction under pseudo-first-order conditions.

Fig. 7 shows $1/\tau_2$ for different TNS concentrations at constant enzyme concentration and for different enzyme concentrations at constant TNS concentration. We used the following reaction scheme assuming a bimolecular mechanism with n equivalent binding sites to describe the kinetic effects:

$$\begin{aligned} E + D &\underset{k_-}{\overset{nk_+}{\rightleftharpoons}} ED \\ ED + D &\underset{2k_-}{\overset{(n-1)k_+}{\rightleftharpoons}} ED_2 \\ &\vdots \quad \vdots \quad \vdots \\ ED_{n-1} + D &\underset{nk_-}{\overset{k_+}{\rightleftharpoons}} ED_n \end{aligned}$$

E: enzyme, D: dye

The following equations hold for the above reaction scheme:[24]

$$1/\tau_2 = k_- + k_+[D] \quad \text{for } [D]_{total} \gg [E]_{total} \qquad \text{(IV)}$$

$$1/\tau_2 = k_- + nk_+[E] \quad \text{for } [nE]_{total} \gg [D]_{total} \qquad \text{(V)}$$

The rate constants k_+ and k_- for the association and dissociation reaction were determined from the slope and intercept of the graph of $1/\tau_2$ as a function of the dye and enzyme concentrations (Fig. 7). Within experimental error equal values for the dissociation rate constants k_- of 1020 ± 300 s^{-1} and 970 ± 40 s^{-1} were obtained from both plots. According to eqn. (IV) and (V) the association rate constants $k_+ = (1.2 \pm 0.2) \times 10^7$ dm^3 mol^{-1} s^{-1} and $nk_+ = (6.5 \pm 0.2) \times 10^8$ dm^3 mol^{-1} s^{-1} were estimated from the graph in Fig. 7. The number of binding sites n was calculated from the ratio of $nk_+/k_+ = 54 \pm 11$. From the ratio k_-/k_+ a value for K_d of $85 \pm 25 \times 10^{-6}$ dm^3 mol^{-1} was obtained.

The binding of the steryl dye RH421 to RUBISCO was detected by fluorescence titrations of the enzyme with RH421,[9] fluorescence lifetime measurements[8] ILTJ experi-

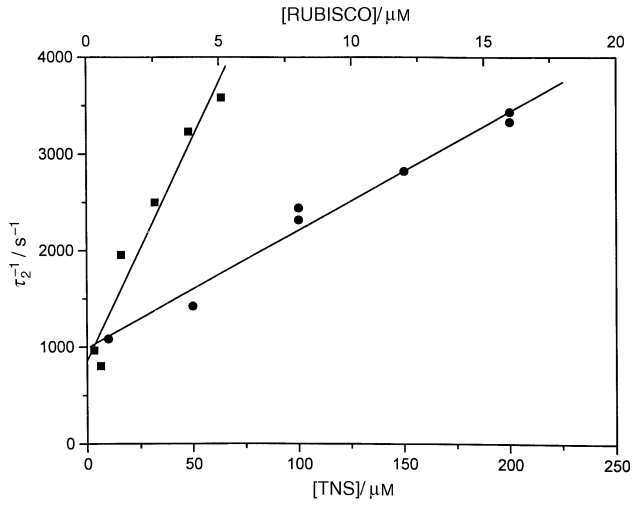

Fig. 7 Reciprocal relaxation times $1/\tau_2$ from ILTJ experiments for the binding of TNS to inactive RUBISCO from spinach as a function of the dye {●, [RUBISCO] = 0.187 µM (0.1 mg ml^{-1})} and the enzyme (■, [TNS] = 10 µM) concentration in the absence of magnesium ions and hydrogen carbonate ions in 20 mM TRIS-HCl buffer pH 8.0 (1 mM dithioerythritol and 0.1 mM EDTA) at 25 °C.

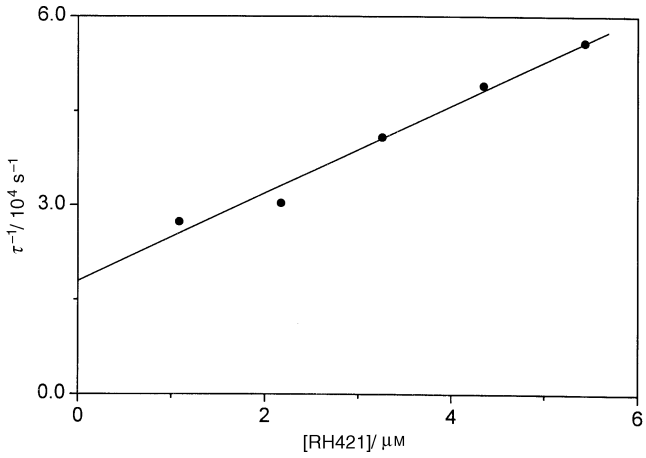

Fig. 9 Reciprocal relaxation times $1/\tau$ from ILTJ experiments for the binding of RH421 to inactive RUBISCO from spinach as a function of the RH421 concentration at a constant enzyme [0.47 µM (0.25 mg ml^{-1})] concentration in the absence of magnesium ions and hydrogen carbonate ions in 20 mM TRIS-HCl buffer pH 8.0 (1 mM dithioerythritol and 0.1 mM EDTA) at 25 °C.

ments. The fluorescence measurements showed a longer lifetime of the bound dye in comparison to the free dye. From fluorescence anisotropy decay measurements a rotational correlation time of 159 ns for the bound dye was found; this is 80% of the rotational correlation time of the enzyme molecule itself.[8] Fluorescence titrations of the inactive RUBISCO from spinach using an excess of RH421 yielded a binding constant $K = (4 \pm 2) \times 10^5$ dm^3 mol^{-1} equivalent to an apparent dissociation constant K_d of $(2.5 \pm 1.3) \times 10^{-6}$ mol dm^{-3}.[9] From the titration of RH421 with an excess of enzyme $nK = (1.8 \pm 0.3) \times 10^6$ dm^3 mol^{-1} was obtained.[9] The number of RH421 binding sites of RUBISCO from spinach was calculated to be $n = 4 \pm 2$.

The binding reaction of RH421 to RUBISCO from spinach showed only one dye dependent relaxation process in ILTJ experiments. A typical relaxation signal is displayed in Fig. 8. Values of $1/\tau$ as a function of the RH421 concentration at constant enzyme concentration is plotted in Fig. 9. The association and the dissociation rate constant $k_+ = (7 \pm 0.6) \times 10^9$ dm^3 mol^{-1} s^{-1} and $k_- = (1.8 \pm 0.2) \times 10^4$ s^{-1} were estimated from the slope and the intercept of the plot in Fig. 9. From these data a dissociation constant $K_d = k_-/k_+ = (2.6 \pm 0.5) \times 10^{-6}$ mol dm^{-3} was calculated.

Discussion

Fluorescence reporter molecules like TNS and RH421 are frequently used to study conformational changes and the reactivity of enzymes by fluorimetry. In previous work we have demonstrated that TNS is an excellent probe for the investigation of substrate and effector binding to RUBISCO,[10a,b] because the enzyme activity is not affected. In a forthcoming publication we shall also demonstrate that the substrate binding at the active site of RUBISCO is not influenced by TNS. In this paper we studied the binding of TNS to the carboxylase of spinach by fluorimetric, microcalorimetric and fast relaxation techniques. By fluorescence titration experiments the binding of TNS to RUBISCO was studied for various activation states of the enzyme. Fluorescence titrations of RUBISCO with TNS and RH421 showed an increase of the fluorescence intensity upon binding of both dyes to the water-soluble enzyme. The concentration dependence of the fluorescence increase at 427 nm for TNS and 648 nm for RH421 allowed us to determine the dissociation constant K_d for the binding of both dyes to RUBISCO. RH421 interacts with RUBISCO from spinach in a different way from TNS showing both a lower apparent dissociation constant K_d and a lower number of binding sites compared to TNS. In the inactive state of RUBISCO in the absence of bicarbonate and magnesium ions $K_d = (2.5 \pm 1.3) \times 10^{-6}$ mol dm^{-3} and $n = 4 \pm 2$ were found for the binding of RH421. For TNS, n was determined for the inactive state of the enzyme by three different methods, (*i*) fluorescence titration experiments, (*ii*) ultrafiltration using Amicon cells and (*iii*) by applying the ILTJ technique for relaxation measurements to obtain kinetic data. Within experimental accuracy we found the number of binding sites $n = 45 \pm 10$. In the inactive state the average value obtained for K_d from (*i*) and (*iii*) was $(90 \pm 15) \times 10^{-6}$ mol dm^{-3}. The results presented in Table 1 demonstrate that a decrease of K_d for the RUBISCO–TNS complex and an increase in the quantum yield of the fluorescence of the bound dye was observed, when the enzyme was activated by preincubation with bicarbonate and magnesium ions or in the presence of the transition-state analogue 2-CABP. The activation of the enzyme does not induce a significant change of the number of binding sites for TNS. Obviously a conformational change of the enzyme during the activation process resulted in tighter binding and reduced mobility of the dye probably due to a higher hydrophobicity of its binding sites. In spite of the higher apparent dissociation constant, TNS is more rigidly bound to RUBISCO than RH421 which can be concluded from polarized fluorescence decay measurements. TNS bound to RUBISCO revealed a single rotational correlation time ϕ of 200 ns. For globular proteins ϕ is related to the molecular weight M by the following equation:[25]

$$\phi = \eta M/RT(v + h) \quad \text{(VI)}$$

η is the viscosity of the solution, R the gas constant and T the absolute temperature; v represents the specific volume of the protein and h is an additional contribution to the volume of the hydrated protein caused by bound water molecules. With a known molecular weight for RUBISCO of 535 000 g mol^{-1} (ref. 26) and typical values for $v = 0.74$ cm^3 g^{-1} (ref. 27) and $h = 0.2$ cm^3 g^{-1} (ref. 25) and a viscosity of the aqueous solution of 0.978 cP (ref. 28) at 293.9 K a rotational correlation time $\phi = 201$ ns was calculated from the above equation. This proves that TNS rotates with the same rotational correlation time as the whole protein. In addition, there is no indication of rapid internal TNS rotation within the protein. The fluorescence anisotropy decay of RH421 bound to RUBISCO was also measured by Visser *et al.* Here the value of the estimated rotational correlation time was 80% of that calculated for the entire RUBISCO molecule.[8] It should be realized, however, that the rotational correlation time obtained for RH421 as probe is much less accurate than in the case of TNS because of the much shorter average fluorescence lifetime of RH421.

Fluorescence lifetime measurements of TNS showed larger average first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ fluorescence lifetimes in the presence of RUBISCO than in its absence. The fluorescence of the free dye showed a fast decay in the picosecond range. For TNS bound to RUBISCO from spinach decay curves in the nanosecond range were recorded. In the presence of magnesium ions, substrate or substrate analogues a decrease of the average first- $\langle\tau_1\rangle$ and second-order $\langle\tau_2\rangle$ fluorescence lifetimes between 10 and 15% was observed for TNS bound to RUBISCO. This result is another indication that TNS acts as environmental probe for the detection of conformational changes and binding events of RUBISCO as observed previously for the binding of substrate and substrate analogues to this enzyme.[10a,b] The decrease of the fluorescence due to ligand binding corresponds to the decrease of the average fluorescence lifetimes in the presence of ligands as observed in fluorescence lifetime measurements.

The kinetics of the binding of TNS and RH421 to RUBISCO were also investigated by ILTJ experiments. Both dyes react in a reversible exothermic bimolecular process with RUBISCO. The binding of RH421 to RUBISCO showed an association rate constant k_+ of $(7 \pm 0.6) \times 10^9$ dm^3 mol^{-1} s^{-1} as is expected for a diffusion-controlled reaction. The following formula is a good estimate of the association rate constant k_{diff}:[29]

$$k_{diff} = [2N_A kT/3\eta][(r_A + r_B)^2/r_{AB}]/r_{AB}^2/(r_A r_B)] \quad \text{(VII)}$$

with the Avogadro number N_A, the Boltzmann constant k, and the radii r_A, r_B, r_{AB} of the reactants and the product, respectively. Assuming values of the radii of RUBISCO and RH421 of 5 and 1 nm and a value for the viscosity of an aqueous solution of 0.891 cP at 298 K,[28] $k_{diff} = 1.3 \times 10^{10}$ dm^3 mol^{-1} s^{-1} was calculated. The association rate constant for the binding of TNS to RUBISCO of $k_+ = (1.2 \pm 0.2) \times 10^7$ dm^3 mol^{-1} s^{-1} is three orders of magnitude lower than k_+ for the binding of RH421. The apparent dissociation constants K_d and the number of binding sites n for the TNS–RUBISCO complex were determined from the kinetic constants. The kinetic data obtained here are in good agreement with those derived from equilibrium measurements. For the interaction of RH421 and RUBISCO there is also a satisfactory agreement between the measured apparent dissociation constant K_d and the corresponding values calculated from kinetic experiments. In conclusion, we found that both TNS

RH421 are very useful probes for the detection of binding events at the active site of RUBISCO, which are otherwise very difficult to follow. Fluorimetric and microcalorimetric analysis of sugar phosphate binding to RUBISCO using these dyes as molecular probes are underway.

References

1 W. O. McClure and G. M. Edelman, *Biochemistry*, 1966, **5**, 1908.
2 W. O. McClure and G. M. Edelman, *Biochemistry*, 1967, **6**, 559.
3 W. O. McClure and G. M. Edelman, *Biochemistry*, 1967, **6**, 567.
4 E. Holler and M. Calvin, *Biochemistry*, 1972, **11**, 3741.
5 W-Y. Lin, C. D. Eads and J. J. Villafranca, *Biochemistry*, 1991, **30**, 3421.
6 D. C. Turner and L. Brand, *Biochemistry*, 1968, **7**, 3381.
7 A. Camerman and L. H. Jensen, *Science*, 1969, **165**, 493.
8 N. V. Visser, A. van Hoek, A. J. W. G. Visser, J. Frank, H-J. Apell and R. J. Clarke, *Biochemistry*, 1995, **34**, 11 777.
9 J. Frank, A. Zouni, A van Hoek, A. J. W. G. Visser and R. J. Clarke, *Biochim. Biophys. Acta*, 1996, **1280**, 51.
10 (*a*) J. Vater and J. Salnikow, *Arch. Biochem. Biophys.*, 1979, **194**, 190; (*b*) J. Vater, J. Salnikow and H. Kleinkauf, *Biochem. Biophys. Res. Commun.*, 1997, **74**, 1618.
11 S. Knight, I. Andersson and C-I. Bränden, *J. Mol. Biol.*, 1990, **215**, 113.
12 G. Bowes, W. L. Ogren and R. Hageman, *Biochem. Biophys. Res. Commun.*, 1971, **45**, 716.
13 G. H. Lorimer, M. R. Badger and T. J. Andrews, *Biochemistry*, 1976, **15**, 529.
14 F. R. Tabita and B. A. McFadden, *J. Biol. Chem.*, 1974, **249**, 3453.
15 F. R. Tabita and B. A. McFadden, *J. Biol. Chem.*, 1974, **249**, 3459.
16 A. van Hoek and A. J. W. G. Visser, *Proc. SPIE*, 1992, **1640**, 325.
17 E. H. W. Pap, P. I. Bastiaens, J. W. Borst, P. A. W. van den Berg, A. van Hoek, G. T. Snoek, K. W. A. Wirtz and A. J. W. G. Visser, *Biochemistry*, 1993, **32**, 13 310.
18 N. V. Visser, A. van Hoek, A. J. W. G. Visser, R. J. Clarke and J. F. Holzwarth, *Chem. Phys. Lett.*, 1994, **231**, 551.
19 C. J. Ho, A. L. Motyka and M. R. Topp, *Chem. Phys. Lett.*, 1989, **158**, 51.
20 J. M. Beechem, E. Gratton, M. Ameloot, J. R. Knutson and L. Brand, in *Topics in Fluorescence Spectroscopy*, ed. J. R. Lakowicz, Plenum, New York, 1991, vol. 2, p. 241.
21 T. Kulinski, A. J. W. G. Visser, D. J. O'Kane and J. Lee, *Biochemistry*, 1987, **26**, 540.
22 T. Wiseman, S. Williston, J. F. Brandts and L-N. Lin, *Anal. Biochem.*, 1989, **179**, 131.
23 J. F. Holzwarth, in *The Enzyme Catalysis Process*, ed. A. Cooper, J. L. Houben and L. C. Chien, Plenum, New York, 1989, p. 383.
24 R. J. Clarke and H-J. Apell, *Biophys. Chem.*, 1989, **34**, 225.
25 J. R. Lakowicz, in *Principles of Fluorescence Spectroscopy*, Plenum, New York, 1983, p. 83; 143.
26 O. H. Knapp, A. N. Qabar and S. N. Vinogradov, *Anal. Biochem.*, 1990, **184**, 74.
27 J. P. Thornber, S. M. Ridley and J. L. Bailey, *Biochem. J.*, 1965, **96**, 29c.
28 R. C. Weast, in *Handbook of Chemistry and Physics*, Chemical Rubber Company Press, Boca Raton, FL, 1987, F37.
29 M. v. Z. Smoluchowski, *Z. Phys. Chem.*, 1917, **92**, 129.

Paper 7/00833J; *Received* 7*th February*, 1997