Role of calcium ions in the structure and function of the di-isopropylfluorophosphatase from *Loligo vulgaris*

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Di-isopropylfluorophosphatase (DFPase) is shown to contain two high-affinity Ca^{2+} -binding sites, which are required for catalytic activity and stability. Incubation with chelating agents results in the irreversible inactivation of DFPase. From titrations with Quin 2 [2-({2-[bis(carboxymethyl)amino]-5-methylphenoxy}-methyl)-6-methoxy-8-[bis(carboxymethyl)-amino]quinoline], a lower-affinity site with dissociation constants of 21 and 840 nM in the absence and the presence of 150 mM KCl respectively was calculated. The higher-affinity site was not accessible, indicating a dissociation constant of less than 5.3 nM. Stopped-flow experiments have shown that the dissociation of bound Ca^{2+} occurs in two phases, with rates of approx. 1.1 and 0.026 s⁻¹ corresponding to the dissociation from the low-affinity and high-affinity sites respectively. Dissociation rates depend strongly on temperature but not on ionic strength, indicating that Ca^{2+} dissociation is connected with conformational changes.

Limited proteolysis, CD spectroscopy, dynamic light scattering and the binding of 8-anilino-1-naphthalenesulphonic acid have been combined to give a detailed picture of the conformational changes induced on the removal of Ca^{2+} from DFPase. The Ca^{2+} dissociation is shown to result in a primary, at least partly reversible, step characterized by a large decrease in DFPase activity and some changes in enzyme structure and shape. This step is followed by an irreversible denaturation and aggregation of the apo-enzyme. From the temperature dependence of Ca^{2+} dissociation and the denaturation results we conclude that the higher-affinity Ca^{2+} site is required for stabilizing DFPase's structure, whereas the lower-affinity site is likely to fulfil a catalytic function.

Key words: Ca^{2+} affinity, Ca^{2+} -binding protein, irreversible inactivation, organophosphates.

INTRODUCTION

The di-isopropylfluorophosphatase (DFPase; EC 3.1.8.2) from the squid, *Loligo vulgaris*, catalyses the hydrolysis of several highly neurotoxic organophosphorus compounds that act as inhibitors of acetylcholinesterase. In addition to degrading di-isopropylfluorophosphate (DFP), the enzyme is active against chemical warfare agents such as cyclosarin ('GF'), sarin ('GB'), soman ('GD') and tabun ('GA'). DFPase activity is found in a variety of tissues in squid; the highest activity is observed in the nervous system, hepatopancreas and salivary gland [1–3]. No natural substrate or physiological role of DFPase has yet been assigned.

DFPase isolated from the head ganglion of *L. vulgaris* is an enzyme of 314 amino acid residues [4] that shows some similarities to other squid-type DFPases because antibodies derived from the DFPase from *Loligo pealei* also show cross-reactivity with the enzyme from *L. vulgaris* [5]. Outside the squid-type DFPase family, no similarity to other proteins is observed [4]. Limited proteolysis of DFPase has shown that the enzyme consists of one compact, stable domain with only one surface loop being accessible to proteolytic cleavage [5a]. Because the two proteolytic fragments remain as a tight complex but are not connected via disulphide bonds it was concluded that the structure is stabilized by extensive non-covalent interactions.

Incubation of DFPase with metal-chelating agents (e.g. EDTA and EGTA) results in the time-dependent irreversible inactivation of the enzyme [6]. The activity could be partly restored by the addition of excess Ca^{2+} , with decreasing recovery as the interval between the addition of EDTA and Ca^{2+} increased. These results

indicated that DFPase requires Ca^{2+} for catalytic activity as well as structural stability.

Calcium binding to proteins has been extensively studied [7–10]. The cation is usually bound to protein carboxylate or amide oxygen atoms with a characteristic six-fold or seven-fold co-ordination geometry. Many proteins are known to be stabilized by the binding of Ca^{2+} to specific sites [10].

For some of these enzymes Ca^{2+} might also have an additional role in the catalytic function. Among many others, structural Ca^{2+} -binding sites have been identified in α -lactalbumin [11], subtilisin [12], α -amylase [13] and the galactose-binding protein of *Escherichia coli* [14]. Secreted phospholipase A₂ is a textbook model for a Ca^{2+} -binding protein in which Ca^{2+} serves as an essential cofactor in electrophilic catalysis, with a role in substrate binding and the chemical step of phospholipid hydrolysis [15,16].

The objectives of the present study were to prove unequivocally the binding of Ca^{2+} to DFPase, to measure the number of Ca^{2+} -binding sites and to determine the affinity for Ca^{2+} as well as the kinetics of Ca^{2+} dissociation. In addition, the effect of the removal of protein-bound Ca^{2+} on the structure and activity of DFPase was analysed in detail. On the basis of the results a potential role of Ca^{2+} in catalysis and in the structural stabilization of the enzyme is discussed.

MATERIALS AND METHODS

Materials

Thrombin and trypsin were from Roche Diagnostics. Competent BL21 cells were purchased from Stratagene. Ni²⁺-nitrilotriacetate

Abbreviations used: ANS, 8-anilino-1-naphthalenesulphonic acid; DFP, di-isopropylfluorophosphate; DFPase, di-isopropylfluorophosphatase (EC 3.1.8.2); DLS, dynamic light scattering; NTA, nitrilotriacetate; PON, paraoxonase (EC 3.1.8.1); Quin 2, 2-({2-[bis(carboxymethyl)amino]-5-methylphenoxy}methyl)-6-methoxy-8-[bis(carboxymethyl)-amino]quinoline; TXRF, total-reflection X-ray fluorescence analysis.

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(Ni-NTA) was from Qiagen. The HiLoad 16/20 Q-Sepharose HP column was purchased from Pharmacia Biotech Europe, the Protein and Peptide column (catalogue no. 218TP52) from Vydac. Chelex 100 was from Bio-Rad. Tricine gels (10–20%) were obtained from Novex. DFP was from Serva GmbH, Sigma-Aldrich Chemie or Fisher Scientific. Vivaspin concentrators were obtained from Millipore. 8-Anilino-1-naphthalenesulphonic acid (ANS), 2-({2-[bis(carboxymethyl)amino]-5-methylphenoxy}methyl)-6-methoxy-8-[bis(carboxymethyl)-amino]-quinoline (Quin 2) (potassium salt) and TbCl_3 were purchased from Fluka Chemie AG. All other chemicals were analytical-grade products from Roth, Sigma-Aldrich Chemie or Fluka Chemie AG.

Expression and purification

DFPase was expressed in BL21 cells transformed with the expression plasmid pKKHisND and purified [5a]. In brief, the enzyme was purified from cell lysates by immobilized metal-affinity chromatography on Ni-NTA, followed by digestion with thrombin, rechromatography on Ni-NTA and anion-exchange chromatography on a HiLoad 16/20 Q-Sepharose HP column. The purified protein was homogeneous as judged by SDS/PAGE, analytical HPLC and HPLC-MS.

Enzyme assay

DFPase activity was measured titrimetrically with a Radiometer 'pH Stat' apparatus [5a]. In this assay DFP hydrolysis was monitored by titrating the released protons with 10 mM NaOH. After correction for the non-enzymic hydrolysis of DFP, the enzyme's activity was obtained in units/ml, where one unit is defined as the hydrolysis of 1 μmol of DFP/min. The specific activity is expressed as units/mg of protein.

Total-reflection X-ray fluorescence analysis (TXRF)

The buffer of protein samples was exchanged for 2 mM Tris/acetate buffer, pH 7.5 [prepared with Millipore water (18 M Ω) and stored in plastic bottles], with Vivaspin concentrators (molecular mass cut-off 10 kDa) that had been extensively washed with Millipore water (18 M Ω) beforehand. The total Ca^{2+} content of the protein solution (10–20 μM) and the residual Ca^{2+} content of the buffer were determined by TXRF.

Metal ion exchange

All vessels used for dialysis were incubated with 2 mM EDTA overnight and afterwards extensively washed with Millipore water (18 M Ω). Protein samples (1–2 mg/ml) were dialysed three times for 24 h against a 100-fold excess of exchange buffer containing 10 mM Tris/HCl, pH 7.5, and either 2 mM MeCl_2 or 2 mM MeSO_4 . For replacement with Zn^{2+} and Cd^{2+} the dialysis buffer contained an additional 1 M NaCl. The metal content of the sample and the protein concentration via the sulphur content of the sample were determined with TXRF. The specific activity was measured as described above.

Fluorescence measurements

The buffer (2 mM Tris/acetate, pH 7.5) used for fluorescence experiments was prepared with Millipore water (18 M Ω) and stored in plastic bottles that had been extensively washed with a 50 mM EDTA solution and Millipore water (18 M Ω) beforehand. For the preparation of Quin 2 stock solutions the buffer was additionally dialysed against a dialysis bag filled with

Chelex 100. Stock solutions were stored at -20°C in the dark and kept on ice during the measurements. Protein samples for fluorescence measurements were brought to the buffer by using Vivaspin concentrators that had been extensively washed with Millipore water (18 M Ω) beforehand, concentrated to approx. 500 μM and stored in plastic vessels at 4°C . The DFPase concentration was determined by using a molar absorption coefficient, ϵ_{280} , of 40 607 $\text{M}^{-1}\cdot\text{cm}^{-1}$. The Ca^{2+} content of the protein samples and that of the buffer were checked via TXRF. All fluorescence measurements with the exception of the stopped-flow experiments were performed with an Aminco Bowman® series 2 fluorescence spectrometer.

ANS binding

The concentration of ANS in the stock solution (approx. 4 mM in 2 mM Tris/acetate, pH 7.5) was determined by using a molar absorption coefficient, ϵ_{350} , of 5000 $\text{M}^{-1}\cdot\text{cm}^{-1}$ [17]. All measurements were performed in 2 mM Tris/acetate, pH 7.5, at 20°C . The emission spectrum of ANS (100 μM) from 400 to 600 nm was recorded in the presence of 10 μM DFPase and a 50-fold excess of either Ca^{2+} or EDTA. ANS fluorescence was excited at 370 nm with a bandwidth of excitation and emission of 4 nm and a sensitivity of 820 V. To follow the kinetics of ANS binding to DFPase during the dissociation of protein-bound Ca^{2+} , 2 ml of 10 μM DFPase was placed in a quartz cell (1 cm $\times$ 1 cm). After temperature equilibrium (25°C) had been reached, 100 μM ANS and 500 μM EDTA were added. The binding of ANS to the protein was followed for 45 min at an emission wavelength of 490 nm, recording 1 data point every 5 s. The excitation wavelength was set to 370 nm and the slit width of emission and excitation was 4 nm. Data were fitted to an exponential function by standard non-linear least-square methods with the program ORIGIN 5.0.

Terbium fluorescence

Equimolar amounts of DFPase (10 μM) and TbCl_3 in 2 mM Tris/acetate, pH 7.5, were mixed in the quartz cell (1 cm $\times$ 1 cm). Measurements were made at 25°C . Terbium was excited by energy transfer from tryptophan residues excited at a wavelength of 292 nm. The emission was scanned at a scan rate of 2 nm/s from 530 to 560 nm, with a bandwidth of emission and excitation of 8 nm, a resolution of 1 nm and a sensitivity of 800 V.

Fluorescence titration with Quin 2

The Quin 2 concentration of the stock solution (approx. 2 mM in Chelex-treated 2 mM Tris/acetate, pH 7.5) was determined on 1:100 dilutions of this stock solution in the presence of Ca^{2+} by using a molar absorption coefficient, ϵ_{240} , of $4.2 \times 10^4 \text{ M}^{-1}\cdot\text{cm}^{-1}$ [18]. Titrations were performed at 25°C in 2 mM Tris/acetate, pH 7.5, with the desired KCl concentration (0, 50, 100 or 150 mM KCl) in a quartz cell (1 cm $\times$ 1 cm). Titrations were performed by the addition of 4 μM aliquots of Quin 2 to 2 ml of a 20 μM DFPase solution. At each titration point the sample was equilibrated for 3 min before measurement of the emission spectrum from 450 to 530 nm. Quin 2 fluorescence was excited at a wavelength of 339 nm. The bandwidth of excitation and emission was set to 4 nm. The emission was scanned at a scan rate of 2 nm/s with a resolution of 1 nm and a sensitivity of 620 V.

Ca^{2+} dissociation constants were extracted from the fluorescence titration data by using the intensity F of the Quin 2 fluorescence, where F is defined as the integral of the fluorescence signal from 450 to 530 nm. Assuming that the increase in fluorescence intensity is proportional to the concentration of the

Ca^{2+} -Quin 2 complex [19], F could be used directly for calculating the dissociation constant. This assumption is justified because the concentration of free Quin 2 is low under the reaction conditions and the fluorescence quantum yield of the Ca^{2+} -Quin 2 complex is approx. 5-fold that of free Quin 2. Because the addition of Quin 2 to the protein solution never exceeded 3% of the total volume, no concentration corrections were made.

The apparent dissociation constant $K_d(\text{app})$ of the Ca^{2+} -Quin 2 complex in the presence of Ca^{2+} -binding protein was calculated from the experimental data by fitting them to the following equation [20] with a standard non-linear least-square program (ORIGIN 5.0):

$$F = (C + x + K_d(\text{app}) - \{[C + x + K_d(\text{app})]^2 - 4xC\}) / [2C / (V - 1)]$$

where C is Ca^{2+} concentration, x is Quin 2 concentration and V is the maximum of F .

Given a Ca^{2+} dissociation constant, K_d , for Quin 2 of 5.3 nM at low salt concentrations (appropriate values were taken for titrations in the presence of KCl) [21], the Ca^{2+} dissociation constant, K_p , of the protein was calculated from:

$$K_d(\text{app}) = K_d(1 + P/K_p)$$

where P denotes the protein concentration.

Stopped-flow protocol

Because Ca^{2+} binding to Quin 2 is very rapid compared to the Ca^{2+} dissociation from a Ca^{2+} -binding protein, the dissociation of Ca^{2+} from the protein was measured with the Ca^{2+} indicator Quin 2 [22].

Stopped-flow experiments were performed at 20 °C, unless stated otherwise, with a HiTech Scientific SF-61 DX2 double-mixing stopped-flow system equipped with a mercury lamp. Measurements at 5–35 °C were included to analyse the temperature dependence of the dissociation of Ca^{2+} . Quin 2 fluorescence was excited with the mercury arc line at 313 nm. Fluorescence was observed at 90° to the incident beam with a 455 nm cut-off filter. In a typical experiment one syringe contained 20 μM protein and additional 20 μM CaCl_2 to ensure full saturation of the Ca^{2+} -binding sites of DFPase, and the other syringe contained 200 μM Quin 2. All solutions were made in 2 mM Tris/acetate, pH 7.5. To determine the salt dependence of Ca^{2+} dissociation, appropriate amounts of KCl (0–150 mM) were added to the buffer.

Data were fitted to an exponential function by non-linear least-square methods with the program Kinet Asyst 2, Version 2.1a (HiTech Scientific). Where necessary a linear function was incorporated into the fitting function to account for photo-bleaching.

Dynamic light scattering (DLS)

All light-scattering experiments were performed with a DynaPro molecular sizing instrument (Protein Solutions). The Dynamics 4.0 software package was used for data analysis. The translational diffusion coefficient (D_t) of the sample particles was determined by measuring the fluctuations in the intensity of scattered light with an autocorrelation function. The hydrodynamic radius (R_h) of the particles was calculated by the Stokes–Einstein equation ($D_t = k_b T / 6\pi\eta R_h$, with k_b being Boltzmann's constant, T the absolute temperature and η the solvent viscosity). Assuming that the particles were spherical and of standard density the molecular mass of the particles was estimated from R_h . As the autocorrelation function was initially analysed with a single exponential cumulant analysis (one particle size), the polydispersity and

error gave an indication of the homogeneity of particle sizes in the solution.

All protein solutions for DLS experiments were filtered through a 0.1 μm filter (Anodisc 13; Whatman). DLS of DFPase (2–5 mg/ml) in 10 mM Tris/HCl (pH 7.5)/2 mM CaCl_2 was measured at 20 °C; 15 independent measurements were acquired for each sample, and the reported values are the calculated arithmetic means.

To analyse the effect of the dissociation of protein-bound Ca^{2+} on the aggregation state of the DFPase, a 100-fold excess of EDTA was added to the protein solution before the DLS experiment. Changes in aggregation state were followed for 1–3 h by accumulating four measurements every 5 min. To analyse the effect of temperature on the observed conformational changes, measurements at 10 °C were included.

CD spectroscopy

CD spectra of DFPase [2 mg/ml in 10 mM Tris/HCl (pH 7.5)/2 mM CaCl_2] were recorded in the far-UV region between 185 and 260 nm at 20 °C on a Jasco J-720 spectrometer equipped with a cell with a 0.01 cm path length. Measurements were taken with a scan rate of 100 nm/s, a response time of 1 s and a resolution of 1 nm and were averaged over 10 scans unless stated otherwise. After the addition of a 100-fold excess of EDTA to the DFPase solution, conformational changes during the dissociation of protein-bound Ca^{2+} were followed for 75 min by accumulating two spectra every 5 min. To analyse the effect of temperature on the observed conformational changes, measurements at 10 °C were included.

Limited proteolysis

Proteolytic digestion of DFPase (0.5–1 mg/ml) with trypsin at a protein-to-protease ratio of 50:1 was performed in 10 mM Tris/HCl, pH 8.0, at room temperature. A 100-fold excess of CaCl_2 or EDTA was added to the protein solution before proteolysis. After the addition of trypsin, samples were taken at several time points and 1 mg/ml Pefobloc® SC was added to inactivate trypsin. The digests were analysed by SDS/PAGE with 10–20% (w/v) Tricine SDS gels.

RESULTS

Calcium binding to DFPase

Previous work on squid-type DFPases indicated that those enzymes bind one or more bivalent cations [6,23]. In the present study the metal-binding characteristics of DFPase and the function of the bound metal ions were further investigated.

The identity and number of bound metal ions were determined by TXRF, showing that DFPase bound 2.1 ± 0.3 Ca^{2+} ions (mean $\pm$ S.D.) even after extensive dialysis against Ca^{2+} -free buffer, indicating a high affinity of the protein for Ca^{2+} .

Kinetics of Ca^{2+} dissociation

The kinetics of Ca^{2+} dissociation from DFPase were measured by complexation with Quin 2 within a range of conditions (ionic strength and temperature). When the protein was mixed with an excess of Quin 2 the kinetics of Ca^{2+} release from the protein were followed by the increase in Quin 2 fluorescence at 495 nm on binding Ca^{2+} . Because the dissociation of Ca^{2+} from DFPase is very slow relative to Ca^{2+} binding by Quin 2 the change in Quin 2 fluorescence is equal to the rate of Ca^{2+} dissociation from the protein.

Figure 1 Rates of Ca^{2+} dissociation from DFPase using Quin 2 fluorescence at 25 °C

Concentrations before rapid stopped-flow mixing were 20 μM DFPase, 20 μM CaCl_2 , 200 μM Quin 2. Quin 2 fluorescence was monitored through a 455 nm interference filter with excitation at 313 nm. Each trace represents a signal average of five to ten traces. The fluorescent transient was measured by Quin 2 for Ca^{2+} dissociation from both Ca^{2+} -binding sites of DFPase. The signal fitted two exponential terms with rate constants of approx. 1.1 s^{-1} and 0.026 s^{-1} . Inset: expanded time scale to permit an accurate measurement of the rate of the fast process; the data were fitted by a single-exponential term (broken line).

Figure 1 shows the time course of the increase in Quin 2 fluorescence that occurred as DFPase was rapidly mixed with a 10-fold excess of Quin 2 at 25 °C in salt free solution. Clearly, two phases with amplitudes of approx. 45% for the faster phase and 55% for the slower phase can be distinguished, which might indicate two distinct Ca^{2+} dissociation processes. It has been reported previously that Quin 2 can interact with proteins, this interaction causing changes in the spectral properties of the dye [24]. To exclude a possible interaction between Quin 2 and DFPase and its influence on the fluorescence properties of Quin 2, changes in Quin 2 fluorescence on mixing with DFPase in the presence of an excess of Ca^{2+} and EDTA were analysed. No changes in the fluorescence properties of Quin 2 were observed under these conditions (results not shown), indicating that the observed fluorescence transient was not caused by interactions between DFPase and Quin 2. Each of the two kinetically distinguishable processes therefore corresponded to the dissociation of one Ca^{2+} ion. The fitted values of the two dissociation rate constants k_{-1} and k_{-2} at 25 °C in salt-free solution were $1.1 \pm 0.1 \text{ s}^{-1}$ and $0.026 \pm 0.002 \text{ s}^{-1}$ respectively. No alterations of

Table 2 Temperature dependence of Ca^{2+} dissociation rates (k) obtained from stopped-flow experiments on DFPase

Values are means $\pm$ S.D. for more than five individual measurements. Subscripts -1 and -2 refer to Ca^{2+} dissociation from the two different Ca^{2+} -binding sites of DFPase.

Temperature (°C)	$k_{-1} (\text{s}^{-1})$	$k_{-2} (\text{s}^{-1})$
5	0.3 ± 0.04	—
10	0.4 ± 0.04	—
15	0.6 ± 0.03	—
20	1.0 ± 0.03	0.023 ± 0.001
25	1.1 ± 0.1	0.026 ± 0.002
30	2.2 ± 0.2	0.034 ± 0.008
35	5.3 ± 0.6	0.047 ± 0.003

Figure 2 Kinetics of Ca^{2+} dissociation from DFPase as a function of temperature

The release of calcium from DFPase was measured by using Quin 2 under conditions identical with those for Figure 1. Shown is a plot of the natural logarithm of the equilibrium constant for the transition state against the reciprocal of the absolute temperature for the fast dissociation process. The straight line represents the fit according to the Eyring equation.

the rate constants with ionic strength in the range 0–150 mM KCl were observed (Table 1).

The two dissociation rate constants were strongly dependent on temperature (Table 2); the rate constant, k_{-1} , of the faster process increased from $0.3 \pm 0.04 \text{ s}^{-1}$ at 5 °C to $5.3 \pm 0.6 \text{ s}^{-1}$ at 35 °C. The plot of $\ln k_{-1}$ against $1/T$ was approximately linear and the data for the fast phase were fitted by the Eyring equation (Figure 2): $\ln(kh/k_B T) = -\Delta H^\ddagger/RT + \Delta S^\ddagger/R$, where h is

Table 1 Kinetic and equilibrium constants for the reaction of Ca^{2+} with DFPase

Values are means $\pm$ S.D. for more than five individual measurements. All experiments were performed at 25 °C as described in the text. Subscripts 1 and 2 refer to Ca^{2+} dissociation from the two different Ca^{2+} -binding sites of DFPase. The dissociation constant, K_i , and the stoichiometry n were obtained from titration curves similar to those in Figure 3, and the dissociation rate constants k_{-1} and k_{-2} from stopped-flow experiments. The association rate constant k_i was calculated from K_i and k_{-1} . Also indicated are the dissociation constants, K_Q , of Quin 2 at different KCl concentrations (from [21]).

KCl (mM)	K_Q (nM)	n	K_i (nM)	$k_{-1} (\text{s}^{-1})$	$k_i \times 10^{-7} (\text{M}^{-1} \cdot \text{s}^{-1})$	$k_{-2} (\text{s}^{-1})$
0	5.3	1.0 ± 0.1	21 ± 4	1.1 ± 0.1	5.1	0.026 ± 0.002
50	40	1.0 ± 0.13	134 ± 23	1.2 ± 0.15	0.9	0.022 ± 0.0018
100	83	1.2 ± 0.1	623 ± 83	1.2 ± 0.24	0.2	0.022 ± 0.0023
150	120	1.1 ± 0.16	842 ± 44	1.2 ± 0.2	0.15	0.021 ± 0.0018

Figure 3 Example of fluorescence titration of Ca^{2+} -DFPase with Quin 2 at 25 °C and low ionic strength

Fluorescence intensity, F , is plotted against concentration of Quin 2. Filled squares are experimental data points; the curve was obtained by least-squares fitting to the results, yielding a Ca^{2+} dissociation constant of DFPase of 21.1 nM. In addition, the data points obtained from the same titration in the absence of protein and Ca^{2+} are shown (●). Inset: spectra recording part of the titration of Quin 2 into 20 μM DFPase in 2 mM Tris/acetate, pH 7.5. The total Ca^{2+} concentration present in the protein solution as determined by TXRF was 41.6 μM , i.e. 1–2 μM free Ca^{2+} . On the addition of 4 μM aliquots of Quin 2 the fluorescence intensity increased and reached a maximum at a DFPase-to-Quin 2 ratio of approx. 1:2.

Planck's constant, k_B is the Boltzmann constant and T is the absolute temperature. The fit yielded values for $\Delta H_1^\ddagger$ of 61 ± 7.2 kJ/mol and $\Delta S_1^\ddagger$ of -0.53 ± 0.36 J/mol K, indicating that the transition state was enthalpically less favourable than the Ca^{2+} -bound state, with only a small change in entropy.

For the slower process no dissociation of the bound Ca^{2+} was observed in the presence of a 10-fold excess of Quin 2 at temperatures of 15 °C or less. At higher temperatures the rate constant k_{-2} for this process increased from 0.023 ± 0.001 s $^{-1}$ at 20 °C to 0.047 ± 0.003 s $^{-1}$ at 35 °C. Because the plot of $\ln k_{-2}$ against $1/T$ was not linear (results not shown), the values obtained for $\Delta H_2^\ddagger$ (approx. 35.2 ± 4.9 kJ/mol) and $\Delta S_2^\ddagger$ (approx. -2.3 ± 0.39 J/mol K) might be inaccurate. The curvature of the plot might have been due to changes in heat capacity associated with the formation of the transition state [25].

Calcium dissociation constants

Because apo-DFPase is not stable (see below), fluorimetric titrations were performed by the stepwise addition of Quin 2 to a solution of Ca^{2+} -complexed DFPase.

Figure 3 shows a typical fluorescence titration together with the optimal curve obtained from least-square fittings to the experimental data points. There was a substantial enhancement of Quin 2 fluorescence on Ca^{2+} binding (Figure 3). An analysis of the raw binding data as described in the Materials and methods section is presented as a plot of F against the concentration of Quin 2 added. From these plots we calculated the reaction stoichiometry and apparent dissociation constants.

The analysis of the titration curves shows that only one of the two protein-bound Ca^{2+} ions was accessible by titration with Quin 2 under all reaction conditions. This was expected from stopped-flow experiments because dissociation of the slower-dissociating Ca^{2+} was not observed at less than a 5-fold excess of Quin 2 (results not shown). This indicated a higher affinity of this Ca^{2+} for DFPase than for Quin 2. The dissociation constant of

Figure 4 Fluorescence emission spectrum of Tb^{3+} bound to DFPase resulting from energy transfer from tryptophan

An equimolar amount of TbCl_3 was added to a 10 μM DFPase solution at 25 °C. After excitation at 292 nm the Tb^{3+} emission spectrum was scanned from 530 to 560 nm at a scan rate of 2 nm/s. Shown are spectra for Tb^{3+} -DFPase (solid line), DFPase in the absence of Tb^{3+} (dotted line) and Tb^{3+} in absence of DFPase (broken line).

this high-affinity Ca^{2+} -binding site must therefore have been less than 5.3 nM at low salt concentrations.

The Ca^{2+} dissociation constant, K_1 , obtained for the lower-affinity Ca^{2+} -binding site of DFPase was 21 ± 4 nM at low salt concentration. The addition of 0.15 M KCl increased K_1 40-fold (Table 1), which corresponds to a 9.2 kJ/mol change in the free enthalpy of binding. This decrease in Ca^{2+} affinity is likely to have been a pure effect of ionic strength because CD spectroscopy did not indicate a change in the protein's structure in the presence of salt concentrations of up to 1 M [5a]. The association rate constant k_1 for the binding of Ca^{2+} to the lower-affinity Ca^{2+} -binding site was calculated from K_1 and k_{-1} by using the equation $k_1 = k_{-1}/K_1$ (Table 1). It therefore seems that the effect of ionic strength on the Ca^{2+} affinity of the lower-affinity Ca^{2+} -binding site of DFPase was almost exclusively due to a decrease in the association rate constant for Ca^{2+} binding.

Terbium fluorescence

Terbium has been used extensively to investigate Ca^{2+} -binding sites in proteins [26–29]. As seen in Figure 4 the addition of equimolar amounts of TbCl_3 to Ca^{2+} -complexed DFPase and excitation at 292 nm resulted in a characteristic fluorescence emission at 545 nm owing to fluorescence transfer from Trp residues close to the terbium-binding site(s) [29,30]. Accurate measurements to analyse the stoichiometry of terbium binding to DFPase were precluded by a partial terbium-induced aggregation of DFPase at higher Tb^{3+} concentrations, which has also been observed for other proteins [28,29,31,32].

Displacement of Ca^{2+} by other metals

The lower-affinity Ca^{2+} bound to DFPase was easily displaced by other bivalent metal ions, namely Sr^{2+} , Mn^{2+} , Ni^{2+} , Co^{2+} , Zn^{2+} and Cd^{2+} , by equilibrium dialysis; the displacement of

Figure 5 Time dependence of the inactivation of DFPase during Ca^{2+} removal

DFPase ($5 \mu\text{M}$) was incubated at room temperature with a 100-fold excess of EDTA (□) as well as with various amounts of Quin 2 at protein-to-Quin 2 ratios of 1:1 (■), 1:5 (▼), 1:20 (●) and 1:40 (▲). Activity was measured at several time points with 5 mM DFP as substrate. The specific activity of DFPase in the absence of EDTA was $213 \pm 10 \mu\text{mol}/\text{min}$ per mg.

Ca^{2+} resulted in only minor changes in the specific activity of DFPase, with activities between 80 % and 104 % of that of Ca^{2+} -DFPase (results not shown). Except for the Zn^{2+} and Cd^{2+} substituted enzyme, the metal substitution had no effect on the stability of the protein. However, DFPase precipitated on dialysis against buffers containing Zn^{2+} or Cd^{2+} under conditions of low ionic strength and the displacement of Ca^{2+} by Zn^{2+} and Cd^{2+} could be achieved only in a solution of high ionic strength (1 M NaCl). This destabilizing effect of Zn^{2+} and Cd^{2+} might have been due to the binding of these ions to non-specific binding sites in the absence of added salt. Such an effect would also explain the Tb^{3+} -induced aggregation (see above).

Effect of Ca^{2+} dissociation on enzyme activity

Incubation of DFPase with a 100-fold excess of EDTA at room temperature decreased DFPase activity by approx. 60 % in 2 h (Figure 5) with the most pronounced decrease in activity (approx. 40 %) occurring during the first 30 min. Even 24 h after the addition of EDTA, DFPase retained 25–30 % of its activity under these reaction conditions. Complete inhibition of DFPase was achieved only after incubating the enzyme with a greater than 500-fold excess of EDTA for more than 10 h (results not shown). No changes in the inactivation rate were observed at lower temperatures (less than 15°C); the maximal decrease in activity of approx. 40 % also occurred during the first 30 min after the addition of EDTA (results not shown).

Similar results were obtained during the incubation of DFPase with different concentrations of Quin 2 (Figure 5): a 50 % inhibition of DFPase was observed after incubation with a 5-fold excess of Quin 2 for only 2 h. Even at a DFPase-to-Quin 2 ratio of 1:1, the activity decreased by approx. 30 % within 2 h, indicating that the primary loss of activity was due to the dissociation of Ca^{2+} from the lower-affinity Ca^{2+} -binding site.

After the depletion of Ca^{2+} from DFPase, the enzyme's activity was only partly restored by the addition of excess Ca^{2+} . The reactivation depended on the time of addition of Ca^{2+} . When Ca^{2+} was added up to 30 min after EDTA, a reactivation of approx. 20 % was observed. Later on, no reactivation could be achieved at all. The irreversible loss of enzymic activity after

Figure 6 Limited proteolysis of DFPase with trypsin in the presence of either Ca^{2+} or EDTA, detected by Tricine/SDS/PAGE

(A) Time course of the proteolysis of DFPase (0.5–1 mg/ml) in the presence of a 100-fold excess of Ca^{2+} . (B) Time course of the proteolysis of DFPase (0.5–1 mg/ml) in the presence of a 100-fold excess of EDTA. Samples were withdrawn at the time points indicated above each lane (where ' stands for minutes). Digestion products were resolved under reducing conditions on a 10–20 % Tricine/SDS/polyacrylamide gel. OPA marks the position of the DFPase. The mobilities of the marker proteins (lane M) are indicated at the left.

prolonged incubation with EDTA indicates that Ca^{2+} dissociation led to irreversible structural changes.

Effect of Ca^{2+} dissociation on enzyme conformation

Because the irreversible inactivation of the DFPase pointed to structural changes associated with the dissociation of the two Ca^{2+} ions bound to DFPase, we analysed the effect of Ca^{2+} on the structure and stability of the protein in detail.

Limited proteolysis

Limited proteolysis can serve as a sensitive probe of protein dynamics to determine conformational changes induced by ligands [33,34]. To establish whether Ca^{2+} stabilized the DFPase

Figure 7 Conformational changes during Ca^{2+} dissociation as revealed by CD spectroscopy

DFPase (2 mg/ml) in 10 mM Tris/HCl containing 2 mM CaCl_2 was incubated with a 100-fold excess of EDTA. CD spectra were obtained at 20 °C and converted to molar ellipticity after averaging and smoothing. (A) Time course of structural changes induced by incubation with EDTA. Spectra were recorded at the time points indicated (in min, shown as °). (B) For a better overview the spectra obtained directly after the addition of EDTA (solid line) and 75 min after the addition of EDTA (dotted line) are shown.

against proteases, the susceptibility of DFPase to proteolytic degradation was assessed in the presence of excess Ca^{2+} or EDTA. The results in Figure 6(A) show only one accessible trypsin cleavage site in the presence of Ca^{2+} and the two fragments remained as a tight complex with no change in enzymic activity [5a]. In contrast, complete degradation of DFPase occurred in the presence of EDTA. As soon as 5 min after the addition of EDTA to a DFPase solution, additional trypsin cleavage sites were partly susceptible, resulting in the formation of polypeptides of approx. 3 and 5 kDa in addition to the fragments already observed in the presence of excess Ca^{2+} (Figure 6B). After 45 min only the lower-molecular-mass fragments remained, indicating a complete denaturation of the enzyme by that time.

CD spectroscopy

The structural changes after the dissociation of Ca^{2+} from DFPase were analysed in more detail with CD spectroscopy (Figure 7). The CD spectrum of DFPase with bound Ca^{2+} was

Figure 8 Time-dependent changes in the aggregation state of DFPase of dissociation of Ca^{2+}

DFPase (2 mg/ml) in 10 mM Tris/HCl containing 2 mM CaCl_2 was incubated with a 100-fold excess of EDTA at 20 °C; EDTA-induced structural changes were followed for 1 h by DLS. The diffusion coefficient, D (◆), the Stokes radius, R_h (○) and the apparent molecular mass (MW) (■) are plotted against the duration of incubation with EDTA. Data were fitted to a single-exponential function, yielding a rate constant for the aggregation process of $8.62 \times 10^{-4} \text{ s}^{-1}$.

characteristic of a largely β -sheet protein with a broad minimum at 209 nm and maxima at 199 and 187 nm. The incubation of DFPase with a 100-fold excess of EDTA at 20 °C resulted in a gradual diminution of the CD spectrum during the first 20–25 min, indicating a decrease in content of secondary structure. Significant alterations in the shape of the CD spectrum were observed between 30 and 75 min after the addition of EDTA. The spectrum at 75 min (Figure 7B) resembled that of a random-coil structure, with a strong negative band at approx. 206 nm. Differences in this spectrum from the classical random coil CD spectrum might have been due to residual structure and/or the formation of aggregates (see below), which might also have stabilized some secondary structure elements. The time constant of the denaturation process was calculated as $(4.8 \pm 0.9) \times 10^{-4} \text{ s}^{-1}$ from the change in ellipticity at 199 nm. It was not possible to make an exact determination of the secondary structure content of DFPase and the changes in secondary structure observed on incubation with EDTA with several programs for the prediction of secondary structure, presumably owing to the protein's high β -sheet content and the variability between the CD spectra of model β -sheet structures.

The structural changes observed during the incubation of DFPase with EDTA depended strongly on temperature. Incubating DFPase with a 100–500-fold excess of EDTA at 10 °C resulted in only minor changes in the CD spectrum for a period of more than 1 h (results not shown).

DLS

To analyse the quaternary structure of DFPase in the presence of excess Ca^{2+} or EDTA, solutions of DFPase were analysed by DLS (Figure 8). DLS analysis showed that DFPase with bound Ca^{2+} is monodisperse with a Stokes radius of 2.51 nm and an apparent molecular mass of $26.8 \pm 0.7 \text{ kDa}$. The difference between the apparent molecular mass and the molecular mass of 35221 Da calculated from the amino acid sequence suggests that the protein deviates slightly from the standard globular shape and density.

The removal of Ca^{2+} from DFPase by incubating the enzyme with an excess of EDTA at 20 °C resulted in a biphasic change

Figure 9 Binding of ANS by DFPase in the presence of Ca^{2+} and EDTA in 2 mM Tris/acetate, pH 7.5, at 20 °C

ANS fluorescence was excited at 370 nm. (A) Emission spectrum of 100 μM ANS in the presence of Ca^{2+} -DFPase (10 μM) (broken line) and 20 min after the addition of 500 μM EDTA to the ANS-DFPase solution (solid line). For reference the intensity of free ANS in 2 mM Tris/acetate, pH 7.5, is also shown (dotted line). (B) Time dependence of the binding of ANS to DFPase (10 μM) in the presence of 500 μM EDTA. The binding of ANS to the protein was followed at an emission wavelength of 490 nm. Fitting the data to a single-exponential function yielded a rate constant for the binding process of $1.2 \times 10^{-3} \text{ s}^{-1}$.

in the aggregation state (Figure 8). Primarily, a particle with a Stokes radius of approx. 3.0–3.1 nm and an apparent molecular mass of 40–45 kDa was formed during the first 25 min after the addition of EDTA. This increase in the Stokes radius and molecular mass indicated changes in the shape and/or density of the protein owing to Ca^{2+} dissociation or the formation of a dimer. After 30 min, higher-molecular-mass aggregates were formed rapidly, with the apparent molecular mass increasing from approx. 74 kDa at 30 min to approx. 490 kDa at 60 min. The high polydispersity index indicates that multiple protein aggregates were formed at this time.

The time constant for the aggregation of DFPase after the dissociation of protein-bound Ca^{2+} was calculated as $(8.6 \pm 0.7) \times 10^{-4} \text{ s}^{-1}$ from the increase in apparent molecular mass and Stokes radius by fitting the data to an exponential function. This time constant was similar to that obtained for the unfolding process observed with CD spectroscopy. In addition, the formation of aggregates observed during the incubation of DFPase with EDTA showed the same temperature dependence

as the changes in secondary structure. When DFPase was incubated with a 100–500-fold excess of EDTA at 10 °C, a monodisperse particle with an apparent molecular mass of 35 kDa was formed and higher-molecular-mass aggregates (100 kDa or more) were not observed until 150 min after the addition of EDTA (results not shown). Those similarities between structural changes and aggregation after the dissociation of Ca^{2+} from DFPase suggests that the transition of apo-DFPase to an unfolded conformation was accompanied by aggregation of the protein, explaining the irreversibility of the denaturation process after prolonged incubation with EDTA.

ANS binding

The hydrophobic fluorescent dye ANS was used as a diagnostic tool to follow the exposure of hydrophobic surfaces during the conformational changes observed on the removal of Ca^{2+} from DFPase. It is evident from Figure 9(A) that Ca^{2+} -bound DFPase had only a low affinity for ANS, as expected for a fully folded protein [35].

After the addition of excess EDTA, the fluorescence intensity increased and reached a steady-state value much higher than that of free ANS (Figure 9B). This curve could be best fitted to a single exponential function. This phenomenon probably reflected an interaction of ANS with Ca^{2+} -free DFPase and was accompanied by a 35 nm blue shift in the emission of the dye from 525 to 490 nm and an intensity enhancement of approx. 3-fold (Figure 9A). The small lag phase seen in Figure 9(B) indicates that the kinetics of the structural changes after Ca^{2+} dissociation was relatively slow. Fitting the data to an exponential function yielded a time constant for the binding of ANS to Ca^{2+} -free DFPase of $(1.2 \pm 0.1) \times 10^{-3} \text{ s}^{-1}$. Thus the binding of ANS to DFPase on the dissociation of protein-bound Ca^{2+} was approximately twice as fast as the unfolding of apo-DFPase and the accompanying aggregation of the protein, indicating that ANS bound to the DFPase species that was observed primarily after the removal of Ca^{2+} , which was characterized by changes in secondary structure and shape compared with Ca^{2+} -bound DFPase.

DISCUSSION

Ca^{2+} binding to DFPase

Previous studies have shown that DFPase is irreversibly inactivated on treatment with metal-chelating agents, indicating that the protein is stabilized by the binding of bivalent cations [6]. The present study demonstrates the presence of two high-affinity Ca^{2+} -binding sites in DFPase.

The Ca^{2+} affinity of Quin 2 was too low to permit a detailed characterization of the site with the higher affinity, i.e. $K_d < 5.3 \text{ nM}$ in the absence of added salt. Because this site was not accessible during titrations with Quin 2 and because the irreversible denaturation of DFPase is attributable to Ca^{2+} dissociation from this site (see below), the use of equilibrium thermodynamics to calculate the Ca^{2+} affinity of the lower-affinity site ($K_d = 21 \text{ nM}$ in absence of added salt) is justified. The high affinity of both Ca^{2+} -binding sites indicates a structural or catalytic function of the Ca^{2+} ions bound to DFPase, because the Ca^{2+} -binding sites are saturated with Ca^{2+} under physiological conditions. A few other proteins with similar high-affinity Ca^{2+} sites have been reported, e.g. α -lactalbumin [11], lactoperoxidase [36], vitamin K-dependent protein S [37] and α -amylase [13]. In those proteins Ca^{2+} is essential for maintaining the protein's structural integrity and might possess an additional catalytic function.

In the presence of 0.15 M KCl the Ca^{2+} affinity of the lower-affinity Ca^{2+} site of DFPase decreases 40-fold. This is consistent with the binding site being in a highly charged environment. Similar salt effects have been found in studies of Ca^{2+} -binding sites of several Ca^{2+} -binding proteins, including calmodulin [38] and calbindin D_{9k} [21], in which the high Ca^{2+} affinity is modulated by a cluster of negative surface charges around the Ca^{2+} -binding sites. The decrease in Ca^{2+} affinity of the lower-affinity site of DFPase is due mainly to a decrease in the association rate constant for Ca^{2+} binding, whereas the dissociation rate constant is essentially unaltered (Table 1). The most likely explanation for this decrease in association rate is the stabilization of the initial Ca^{2+} -protein complex by interactions of the Ca^{2+} ion with negative charges on the protein surface as observed for calbindin D_{9k} [39]. The effect of added salt would be to mask these interactions, resulting in a decreased Ca^{2+} association rate. The independence of the Ca^{2+} dissociation rate of ionic strength might be due to local structural changes in the Ca^{2+} -binding region on Ca^{2+} dissociation, which might be rate-limiting for the dissociation. Consequently, the increased ionic strength of the medium, which would normally lead to an increase in the dissociation rate constant, might be relatively ineffective.

In general, the dissociation of Ca^{2+} from both Ca^{2+} -binding sites of DFPase is rather slow in comparison with regulatory Ca^{2+} -binding proteins such as calmodulin [22] and troponin C [40]. This difference might reflect the fact that those regulatory Ca^{2+} -binding proteins are typically part of a Ca^{2+} messenger system and are designed to be responsive to changes in Ca^{2+} concentration so as to trigger various biological processes. In contrast, in DFPase the Ca^{2+} seems to be an integral part of the protein's structure, which would explain the slow kinetics of Ca^{2+} dissociation. The large contribution of structural factors on the dissociation of Ca^{2+} is confirmed by the temperature dependence of the dissociation process (Figure 2 and Table 2). In particular the dissociation of Ca^{2+} from the higher-affinity binding site strongly depends on temperature and seems to be associated with a change in heat capacity on formation of the transition state, which has been shown to be closely correlated with a change in exposure of hydrophobic surfaces [41]. As already indicated by the non-linearity of the temperature dependence of the dissociation of Ca^{2+} from the higher-affinity binding site, dissociation from this site is accompanied by irreversible structural changes (see below). It is therefore not possible to determine the high-affinity Ca^{2+} dissociation constant from the temperature dependence of the Ca^{2+} dissociation process.

Several factors are known to influence the Ca^{2+} affinity and Ca^{2+} exchange kinetics of proteins, including the charge and static structure of the binding site, the presence of additional charged amino acids on the protein surface near the Ca^{2+} -binding site, the accessibility of the binding site and the structural integrity of the protein's hydrophobic core [42–46]. Thus the results show that the high Ca^{2+} affinity of DFPase is mediated mainly by structural and electrostatic factors.

Effect of Ca^{2+} on DFPase activity and structure

We were able to demonstrate unequivocally that the removal of Ca^{2+} from DFPase results in an irreversible denaturation of the apo-protein, which is slower than the primary Ca^{2+} dissociation. This denaturation is already obvious from the high accessibility of the apo-protein to protease.

The conformational changes after the removal of Ca^{2+} from DFPase were analysed in more detail with CD spectroscopy and

DLS. Those experiments indicated that the conformational changes observed during Ca^{2+} dissociation occur in two distinct steps. The primary slight increase in the apparent molecular mass as well as the exposure of hydrophobic surfaces indicate the formation of a protein form characterized by a loosened tertiary structure, whereas a significant content of regular secondary structure is still present. The formation of this protein form is associated with a loss of enzymic activity that is at least partly reversible. This primary step is followed by an irreversible denaturation step in which the protein unfolds and aggregates.

This irreversible denaturation of DFPase by the complete removal of Ca^{2+} makes it a most unusual Ca^{2+} -binding protein. Calcium is known to stabilize the structure of many proteins, e.g. proteinase K, thermolysin and α -lactalbumin [10,11], but in those proteins the conformational changes observed on Ca^{2+} dissociation are reversed by the addition of an excess of Ca^{2+} . An irreversible denaturation by the removal of Ca^{2+} has been reported for only few proteins, including α -amylase [47] and the hybrid *Bacillus* 1,3–1,4- β -glucanase [48]. A striking similarity is observed between DFPase and the paraoxonase (PON; EC 3.1.8.1) isolated from the serum and liver of several mammals. The complete removal of Ca^{2+} from PON results in an irreversible inactivation of the enzyme [49–51]. According to Kuo and La Du [52], two Ca^{2+} ions are bound to serum PON: one is bound at the active site and the dissociation of this Ca^{2+} results in a reversible loss of enzymic activity; the second ion has a structural role, with the irreversible inactivation of PON being due to the dissociation of Ca^{2+} from this site. For DFPase a structural role of the higher-affinity site is likely because irreversible denaturation as well as the dissociation of Ca^{2+} from this site depend strongly on temperature. Thus irreversible denaturation is clearly associated with Ca^{2+} dissociation from the higher-affinity Ca^{2+} -binding site of DFPase. In contrast, loss of enzymic activity is also observed at lower temperatures and at a protein-to-Quin 2 ratio at which the higher-affinity Ca^{2+} does not dissociate and at which no changes in the protein's structure can be observed via CD spectroscopy and DLS. Consequently, the primary inactivation does not seem to be due to major conformational changes and is likely to originate from Ca^{2+} dissociation from the lower-affinity Ca^{2+} -binding site, which therefore might have a catalytic rather than a structural function. Because the displacement of this Ca^{2+} ion by other bivalent metal ions results in only minor changes in the catalytic activity of DFPase, this putative catalytic function is likely to be a purely electrostatic contribution.

Furthermore, the fluorescence emission spectrum of the Tb^{3+} -DFPase complex (Figure 4) indicates the presence of a Trp residue in the vicinity of the Tb^{3+} / Ca^{2+} -binding site. As only the lower-affinity Ca^{2+} is easily displaced by other metals, it is likely that Tb^{3+} binds to the lower-affinity Ca^{2+} -binding site of DFPase and might be located in the active site of DFPase. Tryptophan residues are located at the active site of the phosphotriesterase from *P. diminuta* [53] and the human serum PON [54], in which these residues have been shown to participate in substrate binding. Therefore tryptophan residues seem to be a general feature of the active site of DFP-hydrolysing enzymes and the putative Trp residue present in DFPase's active site might have a similar role.

In summary, we have shown that DFPase contains two high-affinity Ca^{2+} -binding sites. The results support the conclusion that the lower-affinity Ca^{2+} -binding site is probably required for catalytic activity, whereas the higher-affinity site is essential for maintaining the structural integrity of DFPase. Detailed structural studies on DFPase are in progress to solve the structural basis for the unusual Ca^{2+} -binding characteristics of this protein.

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