PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

Time Course of Chemical and Structural Events in Protein Crystals Measured by Microspectrophotometry [and Discussion]

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Time course of chemical and structural events in protein crystals measured by microspectrophotometry

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The functional properties of proteins in the crystalline state have been investigated over the past 30 years by a variety of methods, including single crystal polarized absorption spectroscopy. This technique has provided information on the accumulation and equilibrium distribution of protein–ligand complexes in the crystal and, in a few cases, on the rates of interconversion of catalytic intermediates. It has been possible to detect synergistic effects in the binding of different ligands, cooperativity and half-site reactivity and even formation of active multiprotein complexes, obtained by diffusion of one small protein in the pre-formed crystals of the other. Lattice interactions restrain the conformational transitions of some proteins existing in multiple states in solution. The crystal offers the unique opportunity to analyse not only the structure but also the function of a single form of the protein. The relevance of these data to the planning and interpretation of structural studies, especially in the perspectives of time-resolved crystallography, will be discussed with reference to well-characterized systems.

1. Introduction

Proteins in the crystalline state maintain some of their *in vivo* functional properties, including binding specificity and catalytic power (Rupley 1969). However, quantitative studies on a few proteins suggest that intermolecular interactions might alter their native structure and limit their dynamics. To draw correct mechanistic inferences from an X-ray crystallographic study, it is thus necessary to verify whether, or to what extent, the crystalline protein and its ligand-containing derivatives exhibit the properties of the corresponding species in their physiological environment or in solution.

Useful criteria to compare critical structural features as well as function in different physical states are the characteristic electronic spectra of protein-bound chromophores, including prosthetic groups, coenzymes, substrates and analogues, and the equilibrium and kinetic constants determined from ligand and time-dependent spectral changes. For single crystals, accurate spectroscopic data can be obtained by polarized absorption microspectrophotometry. The prerequisite for measuring catalytic rates is that reactions be initiated synchronously and uniformly throughout the crystal. This condition has been achieved either by crystallizing a slow-reacting enzyme–substrate intermediate (Rossi & Bernhard 1970) or by using poor substrates and analogues that diffuse through the crystal liquid channels to

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reach the equilibrium concentration in a time short in comparison with the half life of reaction (Rossi *et al.* 1978; Mozzarelli *et al.* 1979; Vas *et al.* 1979). Some of the reactions we have studied involve, in solution, conformational changes that allow for allosteric interactions, including negative and positive cooperativity.

In this paper, we shall summarize the results of some of our earlier single crystal microspectrophotometric measurements with the hope of stimulating an interest in carrying out corresponding time-resolved crystallographic studies. Furthermore, we shall report recent data on tryptophan synthase from *Salmonella typhimurium* and human haemoglobin, to emphasize the relevance of a detailed spectroscopic investigation of crystalline proteins both in the planning and in the interpretation of crystallographic experiments.

2. Materials

Crystals of α-chymotrypsin (space group $P2_1$) and O-[β-(3-indole)acryloyl]-α-chymotrypsin were grown at 20 °C from concentrated ammonium sulphate solutions containing 3% (by volume) dioxane, 50 mM citrate, pH 4 (Rossi & Bernhard 1970). Tetragonal crystals of γ-chymotrypsin (space group $P4_22_12$) were grown at 20 °C from unbuffered concentrated ammonium sulphate solutions (Merli & Rossi 1986). Both α and γ-chymotrypsin were commercial enzymes from Worthington.

Glyceraldehyde-3-phosphate dehydrogenase was purified from the tail muscle of the mediterranean lobster *Palinurus vulgaris*. Crystals (space group C2) were grown at 4 °C from a concentrated ammonium sulphate solution containing 0.5 mM NAD^+, 1 mM EDTA, 1 mM 2-mercaptoethanol, pH 6.2 (Berni *et al.* 1977, 1979). For reactivity studies, the crystals were transferred to 70% saturated ammonium sulphate solutions at pH 7. Orthorhombic crystals of the Atlantic lobster *Homarus americanus* enzyme were a gift from Professor Michael Rossmann. Monoclinic crystals of the *Bacillus stearothermophilus* enzyme were a gift from Dr Alan Wonacott.

Crystals of pig heart mitochondrial aspartate aminotransferase were obtained at 20 °C by vapour diffusion in hanging drop from a 17% (by mass) polyethylene glycol 4000 M_r solution, 10 mM glycine/NaOH buffer, pH 9.1. Experiments were usually carried out at 25 °C, after suspending crystals in a solution containing 30% (by mass) polyethylene glycol 4000 M_r and 0.2 mM pyridoxal-5′-phosphate to saturate the coenzyme binding sites (Rossi *et al.* 1978; Mozzarelli *et al.* 1979).

Monoclinic crystals of tryptophan synthase from *Salmonella typhimurium*, strain TB2211 (space group C2), were grown by vapour diffusion in sitting drop of a solution containing 6% (by mass) polyethylene glycol 8000 M_r, 1 mM dithiothreitol, 0.01 mM pyridoxal-5′-phosphate, 0.025% NaN_3, 50 mM sodium N,N-bis(2-hydroxyethyl)glycine, 1 mM EDTA, 1.4 mM spermine, pH 7.8, with a reservoir containing 12% (by mass) polyethylene glycol 8000 M_r, 50 mM sodium N,N-bis(2-hydroxyethyl)glycine, 1 mM EDTA, 0.04% NaN_3, at 24 °C (Ahmed *et al.* 1985). For polarized absorption measurements crystals were resuspended in a solution containing 50 mM sodium N,N-bis(2-hydroxyethyl)glycine, 1 mM EDTA, 1.5 mM spermine, 20% (by mass) polyethylene glycol 8000 M_r (Mozzarelli *et al.* 1989).

Orthorombic crystals of human haemoglobin (space group $P2_12_12$) were grown by mixing in different ratios a deoxygenated haemoglobin solution containing 10% (by mass) protein, 10 mM potassium phosphate, 1 mM EDTA, pH 7.2, with a solution containing 10 mM potassium phosphate, 36% (by mass) polyethylene glycol 8000 M_r, pH 7.2, in the absence of oxygen and in the presence of 30 mM sodium dithionite

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(Ward *et al.* 1975; Mozzarelli *et al.* 1991*a*). For polarized absorption measurements, crystals (10–30 μm thick) were transferred anaerobically to a medium containing 10 mM potassium phosphate, 36% to 62% (by mass) polyethylene glycol 8000 M_r, pH 7.2.

3. Methods

(*a*) *Microspectrophotometric measurements*

The extinction coefficients of oriented chromophores bound to crystalline proteins are different for different crystal directions. Consequently, absorption spectra must be measured using linearly polarized light. If the electric vector is parallel to a principal optical direction, it remains linearly polarized as it propagates through the crystal, a necessary condition for the Lambert's law of exponential attenuation of intensity to hold. The isotropic extinction coefficient can be calculated from the polarized extinctions as $\epsilon = \frac{1}{3}(\epsilon_a + \epsilon_b + \epsilon_c)$, where a, b and c are the principal optical directions of the crystal.

For orthorhombic crystals, as those of human haemoglobin grown from a polyethylene glycol solution, the a, b and c principal optical directions coincide with the crystal axes. The polarized absorption spectra of haemoglobin were recorded with light incident on the [010] ac face. A comprehensive description of polarized absorption spectroscopy of haemoglobin has been published by Eaton & Hofrichter (1981).

In the case of monoclinic crystals of tryptophan synthase, we measured absorption of linearly polarized light with its electric vector parallel to the principal optical directions a and b of the [210] face. Due to crystal morphology, only rarely were we able to measure absorption along the c direction.

Absorption values are determined as $A = \log_{10}(I_0/I)$, where I and I_0 are the intensities of monochromatic light transmitted, respectively, by the crystal and by the suspending medium.

Single crystals mounted in a flow cell are placed on a thermostatted stage of a modified Zeiss MPM03 microspectrophotometer. In most experiments, we used 10× Zeiss Ultrafluar UV objectives both as the condenser and the superstage objective. The monochromatic light beam, polarized using a Glan–Thompson prism, is doubly masked with the image of a field diaphragm and a circular mask (5–20 μm in diameter) at the image plane of the superstage objective.

Two types of flow cells are used: (i) quartz windows, separated by either polyethylene or glass walls, 0.1–0.2 mm thick, enclosing a volume of about 30 μl. The cell upper window can be easily removed for mounting and handling individual crystals. Channels through the walls allow replacement of the suspending medium with a new solution in about 30 s; (ii) a gas-tight Dvorak–Stotler cell (Dvorak & Stotler 1971) for binding and kinetic measurements that involve gases. Crystals, mounted on the bottom window of the flow cell, are covered by a gas permeable membrane that does not depolarize linearly polarized light (Gill 1981). The cell is then closed and equilibrated with the desired gas atmosphere. Humidified gas mixtures are prepared by using commercial or home-made devices. The gas partial pressure can be constantly monitored using the appropriate electrodes before or after the flow cell. In the case of oxygen, Clark electrodes have been generally used. The inside chamber has a volume of about 0.3 ml and, with a gas flow of about 50 cm^3 min^{-1}, gas exchange requires a few seconds. However, gas equilibration of the crystal suspending medium is slow (5–10 min) and dependent on its thickness.

Therefore, only slow kinetics can be measured and precise saturation measurements are time consuming, since each data point requires 30–60 min.

(b) *Reaction initiation*

For equilibrium and kinetic measurements, reaction was initiated by replacing the crystal suspending medium with a new medium of similar composition containing the desired reagent. Equilibration between the outside medium and the crystal liquid channels has been shown to be relatively fast and complete within the time required to start measurements. Moreover, since the number of protein binding sites in the crystal is negligible compared with the total number of reagent molecules available in the flow cell, the reagent concentration remains practically constant in the course of binding or single transient reactions. Accordingly, bimolecular reactions between crystalline enzyme and substrates or other reagents follow pseudo-first order kinetics.

(c) *Detection*

Binding and chemical reaction between protein and reagents lead to accumulation, within the crystal, of protein derivatives that represent an appreciable fraction of the total protein concentration. Given the extinction coefficients of the various chromophores involved in these reactions, and typical thickness of crystals, absorbances ranging from 10^{-2} to 2 units are expected, that can accurately be measured with our instrument. Depending on the reaction rate, we can follow kinetics either at a single wavelength or by recording spectra in a wide wavelength range.

(d) *Data analysis*

When mixtures of several species accumulate in the crystal as a result of reaction, various procedures can be used to determine the concentration of the individual species. The method we are using requires the knowledge *a priori* of the spectrum of the individual species present in the mixture. Then, the time or ligand concentration dependent spectral changes can be least squares fitted to a linear combination of such reference spectra (Mozzarelli *et al.* 1991*a*). Other methods can be reliably used (Kallen *et al.* 1985; Metzler *et al.* 1988; Henry & Hofrichter 1992).

4. Results

(a) *α and γ chymotrypsin*

Microspectrophotometry was used to measure enzyme kinetics in single crystals probably for the first time by Rossi & Bernhard (1970). The aim of this study was to compare structure and function of α-chymotrypsin in the crystal and in solution, on the basis of the spectrum and the reactivity of the chromophoric acyl-enzyme analogue O-[β-(3-indole)acryloyl]-α-chymotrypsin. The covalent enzyme derivative had been prepared in solution and, due to its stability at pH 4, had been crystallized in the usual mother liquor of α-chymotrypsin. The characteristic spectrum of this derivative is distinct from that of O-acyl-ester model compounds and is diagnostic of its reactivity state towards hydrolysis. In fact, in denaturating solvents, the acyl-enzyme acquires the spectrum of model compounds and is chemically inert. In water, the pseudo first-order rate constant of deacylation is pH-dependent and the deacylation rate-pH profile fits a sigmoidal curve with $k_{max} = 0.07$ min^{-1} and pK = 7.6.

In the crystal, the acyl-enzyme was found to exhibit the spectrum of the active

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species in solution. This finding was consistent with the hypothesis that its conformation in the crystal, determined by X-ray crystallography (Henderson 1970), is the same as in solution. In fact, by changing the pH of the suspending medium, within the range of crystal stability (pH < 6), and by following the time course of the spectral transition associated with hydrolysis of the acyl bond and formation of the indoleacrylate product, deacylation was found to occur with the same rate as in solution.

In latter experiments, Merli & Rossi (1986) used the more stable crystals of γ-chymotrypsin, to extend measurements of the rates of deacylation to the pH range 6–10 and found that the maximal rate was the same as in solution. However, for this species, an effect of crystallization on the geometry of the active site was revealed by a shift of 0.9 pH units, with respect to solution, of the deacylation rate-pH profile.

(*b*) *Glyceraldehyde-3-phosphate dehydrogenase*

The use of chromophoric reagents to prepare covalent enzyme derivatives that are metastable analogues of true catalytic intermediates was extended by Sidney Bernhard and his co-workers to investigate some aspects of the catalytic and regulatory mechanism of the tetrameric enzyme glyceraldehyde-3-phosphate dehydrogenase (Malhotra & Bernhard 1968, 1973). In solution, the reaction of β-(2-furyl)acryloyl phosphate with NAD^+-saturated muscle or yeast glyceraldehyde-3-phosphate dehydrogenase to form the β-2-furylacryloyl-enzyme exhibits negative cooperativity, or 'half-of-the-sites' reactivity. However, the di-acyl-enzyme derivative exhibits some functional properties similar to those of the natural intermediate 3-phosphoglyceroyl-enzyme. In particular, it requires NAD^+ bound to the acylated sites for phosphorolysis or arsenate assisted hydrolysis of the acyl bond to occur.

We exploited the large spectral changes of the furylacryloyl group associated with its transfer to, or from, the sulphydryl groups of the active site cysteines to monitor the time course of accumulation and decay of the acyl-enzyme (Berni *et al.* 1977; Vas *et al.* 1979; Mozzarelli *et al.* 1982). Since sulphate ions, a component of the crystal suspending medium, strongly inhibit enzyme activity, diffusion was expected to be comparatively fast with respect to chemical reactions. Most of our spectroscopic studies were carried out using monoclinic crystals of the enzyme from the tail muscle of the Mediterranean lobster *Palinurus vulgaris* (Berni *et al.* 1979) but qualitatively similar results were obtained using crystals of the enzyme from the tail muscle of *Homarus americanus*, the structure of which had been determined (Moras *et al.* 1975).

The time course of acylation, dependent on the concentration of the acylating reagent, was found to be the same as in solution in the presence of a comparable concentration of sulphate ions. The reaction appeared to be at least biphasic. For the fast phase, accounting for most of the amplitude of the spectral change, the second-order rate constant was calculated to be 50 ± 5 M^{-1} min^{-1}. The slow phase was due to acylation beyond the two fast reacting sites (Vas *et al.* 1979).

In solution, the spectrum of β-(2-furyl)acryloyl glyceraldehyde-3-phosphate dehydrogenase is remarkably affected by the presence of NAD^+ bound to the acylated sites. The 'inert' apo-acyl-enzyme has a spectrum centred around 340 nm, as model derivatives, while the NAD^+-saturated acyl-enzyme, fully reactive with phosphate or arsenate, has a spectrum centred around 360 nm. A characteristically perturbed spectrum is therefore associated with the active state of the acyl-enzyme. In the crystal, spectrum and reactivity of the acyl-enzyme were similarly NAD^+-

dependent. For NAD^+-saturated acyl-enzyme, the second-order rate constant was found to be 75 M^{-1} min^{-1}, close to the value determined for deacylation in solution, in the presence of nearly as high ammonium sulphate concentrations. The activation of the apo-furylacryloyl enzyme is a kinetically complex process, both in solution and in the crystal, and depends on a protein isomerization (Mozzarelli *et al.* 1982).

(*c*) *Aspartate aminotransferase*

Crystals of aspartate aminotransferase from various sources, in their native form and in the presence of various substrates and analogues have been the object of thorough crystallographic and related spectroscopic studies for several years (Eichele *et al.* 1978; Metzler *et al.* 1978; Rossi *et al.* 1978; Mozzarelli *et al.* 1979; Makarov *et al.* 1980, 1981; Arnone *et al.* 1984; Vincent *et al.* 1984; Metzler *et al.* 1988). The spectral properties of pyridoxal-5′-phosphate and its derivatives formed in the course of catalysis allow a detailed analysis of the nature and the relative amounts of intermediates accumulating at equilibrium in the presence of one or more reagents.

Single crystal microspectrophotometric studies on the pig heart mitochondrial isoenzyme allowed us to determine not only the equilibrium dissociation constants for the four natural substrates of the enzyme (L-glutamate, 2-oxoglutarate, L-aspartate and oxaloacetate) and some substrate analogues, but also the single transient rates of amino group transfer to and from the slow reacting substrates pyruvate and alanine (Rossi *et al.* 1978; Mozzarelli *et al.* 1979). The rates of these processes were unaffected by the comparatively much faster diffusion of reagents and were found to be essentially the same in the crystal and in solution. For pyruvate, the second-order rate constant was about 100 M^{-1} min^{-1}. For L-alanine, the rate constant was 0.7 M^{-1} min^{-1}. Moreover, the rate of alanine transamination linearly increased, as in solution, with increasing concentrations of formate.

In a later study, that was carried out to determine the retention in the crystalline state of some characteristic functional and structural differences between the active sites of cytoplasmic and mitochondrial aspartate aminotransferase, we investigated the reaction of the two apo-enzymes with the coenzyme derivative 4′-N-(2,4-dinitro-5-fluorophenyl) pyridoxamine-5′-phosphate. In the crystal, as in solution, both apo-enzymes were able to cleave the derivative to produce pyridoxal-5′-phosphate and generate a catalytically active holo-enzyme; in both states the cytoplasmic isoenzyme was also able to catalyse an alternative reaction path leading to formation of a covalent bond between the active site lysine and the C-5 of the 2,4-dinitrophenyl moiety of the reagent (Ottonello *et al.* 1983). The temporal resolution of this slow reaction might illustrate some subtle structural or dynamic differences between the active sites of the two isoenzymes.

(*d*) *Tryptophan synthase*

Tryptophan synthase is a tetrameric $\alpha_2\beta_2$ complex catalysing the final reactions in the biosynthesis of L-tryptophan (Yanofsky & Crawford 1972; Miles 1979, 1991).

At the α-sites, indole-3-glycerol phosphate is cleaved to produce indole. At the β-sites, L-serine reacts with pyridoxal-5′-phosphate to generate a sequence of intermediates, including the gem-diamine, the external aldimine, the quinonoid, and the α-aminoacrylate Schiff base. Indole is channelled through the macromolecule to the β-sites, where it reacts to form, via a quinonoid intermediate, L-tryptophan. The α- and β-site reactions are mutually regulated and, since the two sites are 20–25 Å†

† 1 Å = 10^{-10} m = 10^{-1} nm

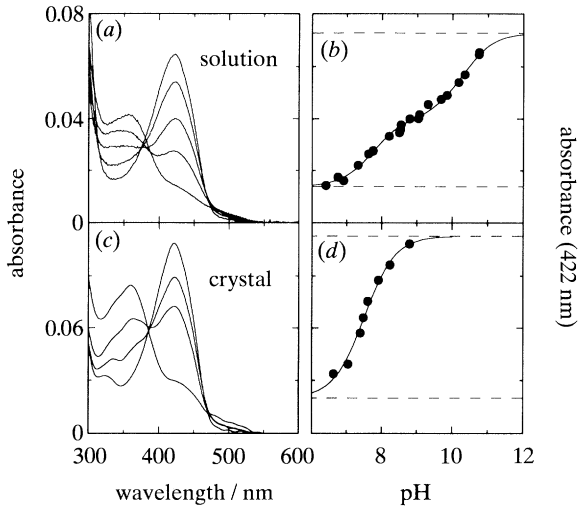

Figure 1. Effect of pH on the distribution of intermediates accumulating in the reaction of L-serine with tryptophan synthase from *Salmonella typhimurium*, in the crystal and in solution. (*a*) Absorption spectra of the enzyme in solution in the presence of 100 mM L-serine at pH 6.40, 7.75, 8.79, 10.18, 10.74, at 20 °C. The intensity of the 422 nm band increases with pH. (*b*) The values of absorbance at 422 nm from a series of spectra, including those shown in (*a*), were least squares fitted to the equation for the titration of two acid residues with apparent pK_a values of 7.7 ± 0.1 and 10.3 ± 0.1. The ratio of the amplitudes is 0.82. This parameter is temperature dependent (Mozzarelli *et al.* 1991*b*). The dash lines are the extrapolated absorbances at low and high pH. (*c*) Polarized absorption spectra of enzyme crystals suspended in buffer solutions containing 20% (by mass) polyethylene glycol 8000 M_r, 50 mM L-serine, 1.5 mM spermine at pH 6.63, 7.60, 7.90, 8.80, at 20 °C. Light was polarized parallel to the principal optical direction along which the extinction coefficients of external aldimine and aminoacrylate are maximal (Mozzarelli *et al.* 1989). (*d*) The values of absorbance at 422 nm from a series of spectra, including those shown in (*c*), were least squares fitted to the equation for the titration of one acidic residue with apparent pK_a value of 7.5 ± 0.1.

apart, the control is purely allosteric. Each catalytic intermediate is characterized by a distinct absorption spectrum and, presumably, by a distinct conformation (Houben & Dunn 1990). The structure of the enzyme from *Salmonella typhimurium* (strain TB2211) has been determined (Hyde *et al.* 1988). Microcrystals of the enzyme are catalytically active and do not break in the course of steady-state reactions (Ahmed *et al.* 1987).

We have compared the polarized absorption spectra of crystals of the native enzyme and of its derivatives, formed in the presence of either L-serine alone or L-serine plus an α-site substrate-analogue, with the corresponding spectra in solution (Mozzarelli *et al.* 1989). A new and unexpected finding was that the equilibrium distribution of intermediates accumulating in the presence of L-serine is pH-dependent (figure 1*c*,*d*). A somewhat different pH-dependence was found in solution (figure 1*a*, *b*; Mozzarelli *et al.* 1991*b*). The tautomer of α-aminoacrylate absorbing at 350 nm (Drewe & Dunn 1985) prevails at low pH, while the external aldimine, absorbing at 422 nm, prevails at high pH. Furthermore, as expected from the known solution behaviour of the enzyme, the α-aminoacrylate intermediate is strongly favoured with respect to the external aldimine by the presence of the α-subunit ligand DL-α-glycerophosphate (Mozzarelli *et al.* 1989). Similarly, a metastable quinonoid, formed by reaction of α-aminoacrylate with mercaptoethanol, is strongly

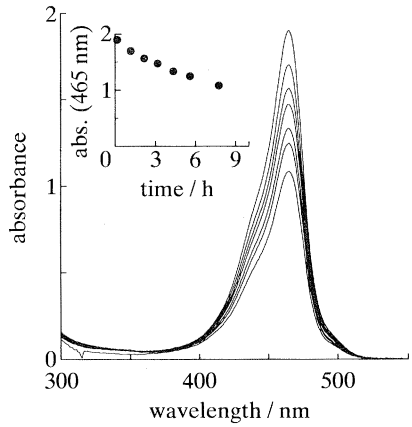

Figure 2. Time course of decay of the quinonoid intermediate formed in the reaction of crystalline tryptophan synthase from *Salmonella typhimurium* with L-serine and indoline. Time-dependent polarized absorption spectra of the quinonoid species formed in the reaction of enzyme with 50 mM L-serine and 10 mM indoline, in a buffer solution containing 20% (by mass) polyethylene glycol 8000 M_r, 1 mM EDTA, 1.25 mM spermine, pH 7.8. Between spectra recordings, the crystal was kept in the dark since the quinonoid species is photolabile. Inset: the absorbance decay at 465 nm indicates a half life of about 6 hours, as in solution (Roy *et al.* 1988).

stabilized, in the crystalline state as in solution, by the α-subunit ligand (Mozzarelli *et al.* 1989). By use of other substrates and analogues, we have also trapped gem-diamine intermediates (Mozzarelli *et al.* 1989) and quinonoids that accumulate in considerable amounts, even in the absence of α-subunit ligands, and are stable enough for structural investigations (figure 2). These findings are useful to define experimental conditions in which either a single species predominates in the crystal, or two or more are present in known proportions.

(*e*) *Human haemoglobin in the T quaternary state*

Human haemoglobin crystallized from solutions of polyethylene glycol and transferred to a medium with a higher concentration of the precipitating agent was found to maintain the quaternary structure of deoxyhaemoglobin even in air, where it appeared to bind oxygen to the α, but not to the β subunits, without breakage of salt bridges (Brzozovski *et al.* 1984; Liddington *et al.* 1988). These crystallographic findings prompted us to apply single crystal polarized absorption spectroscopy to investigate quantitatively oxygen binding to haemoglobin within the rigid frame, created by lattice interactions, that prevents the T to R quaternary transition. It should be noted that crystals of deoxyhaemoglobin grown in concentrated salt solutions crack when exposed to oxygen (Haurowitz 1938; Perutz *et al.* 1964).

Polarized absorption spectra were recorded with the electric vector parallel either to the *a* or to the *c* axis of thin orthorombic crystals in the presence of increasing oxygen pressures (figure 3*a*,*c*). The fractional saturation of haemoglobin as a function of oxygen partial pressure was obtained from the observed spectra after subtraction of the contribution of methaemoglobin and calculation of the ratio between the oxygenated and the total reduced (deoxygenated plus oxygenated) haemoglobin. The fitting of the data by a linear combination of the reference spectra corresponding to deoxygenated, oxygenated and oxidized haemoglobin was very good (Mozzarelli *et al.* 1991*a*). The resulting oxygen binding curves, shown in figure

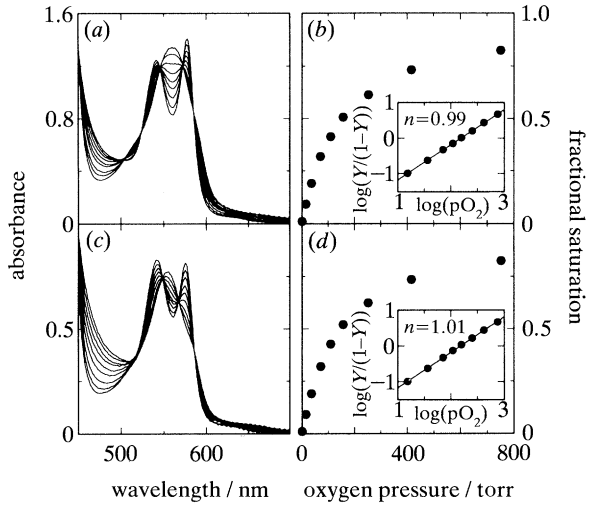

Figure 3. Spectra and oxygen binding curves of T-state human haemoglobin in the crystal. Haemoglobin crystals, suspended in 62 % (by mass) polyethylene glycol 8000 M_r, 10 mM potassium phosphate, pH 7.2, at 15 °C, were equilibrated with oxygen pressures varying between 0 and 760 Torr. Spectra were recorded at each oxygen pressure with light polarized parallel either (*a*) to the *a* or (*c*) to the *c* crystal axis. Oxygen fractional saturations (*b*) and (*d*) were calculated by fitting the observed spectra with a linear combination of the polarized absorption spectra of deoxyhaemoglobin, methaemoglobin and oxyhaemoglobinin in the T-state, plus a baseline (Mozzarelli *et al.* 1991*a*). Insets: Hill plots of the data. (*b*) p50 = 155 Torr, (*d*) p50 = 141 Torr.

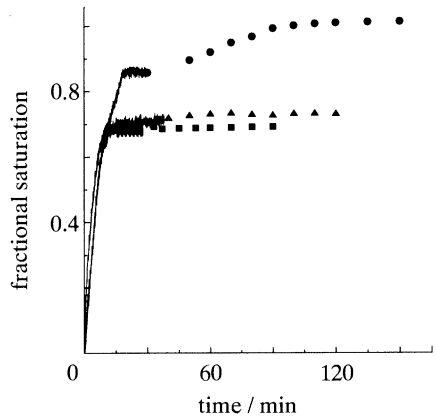

Figure 4. Time course of oxygenation of haemoglobin crystals. Crystals of deoxygenated haemoglobin were exposed to a gas flow (50 cm^3 min^{-1}) of 160 Torr of oxygen, at 8 °C. The time course of oxygenation was monitored for haemoglobin crystals initially containing either 2 % methaemoglobin (●) or 14 % methaemoglobin (▲), suspended in 36 % (by mass) polyethylene glycol 8000 M_r, 10 mM potassium phosphate, pH 7.2, and for crystals containing 3 % metHb, suspended in 54 % (by mass) polyethylene glycol, 10 mM potassium phosphate, pH 7.2 (■). The oxygenation process was followed in the fast phase by recording the absorption changes at 560 nm, and in the slow phase by recording complete spectra between 450 and 700 nm.

3*b*,*d*, represent true equilibrium binding, since they are fully reversible and indicate that fractional saturations of the order of 80 % can be reached in intact crystals. The Hill plots of the data show that n is very close to one (figure 3*b*,*d*, insets). Therefore, the first model-related finding of these measurements is that within the T state in the

crystal there is no immediate evidence for cooperativity in oxygen binding, as predicted by the Monod, Wyman and Changeux model (Monod *et al.* 1965). However, the different p50 values determined from measurements with light polarized parallel either to the *a* or to the *c* axis, respectively 155 and 141 Torr† at 15 °C, reflect a somewhat higher affinity for oxygen of the α haems with respect to the β haems. This difference in relative affinities could be estimated by taking into account the respective contributions of α and β haems to the *a* and *c* polarized absorption spectra (Mozzarelli *et al.* 1991*a*).

Another important observation, derived from spectroscopic studies, is that the oxygen affinity of haemoglobin in the crystal is constant over the pH range 6 to 8.5 (Mozzarelli *et al.* 1991*a*). This finding is predicted by Perutz's stereochemical mechanism concerning the central role of salt bridges in the Bohr effect (Perutz 1970).

A further interesting phenomenon is the change in the polarization ratio going from the fully deoxygenated to fully oxygenated haemoglobin (Mozzarelli *et al.* 1991*a*). The polarization ratio is defined as the ratio of absorptions of light polarized parallel to the *a* and to the *c* crystal axis and is only dependent on the orientation of the haems with respect to the crystallographic axes. A variation in this parameter as a consequence of oxygenation suggests a change in haems orientation and, since the haems are tightly packed within the protein, an associated tertiary conformational change.

All the results reported until now pertain to crystals that do not crack upon oxygenation. In other experiments, we raised the partial pressure of oxygen from 0 to 160 Torr in a single step and measured the kinetics of oxygen binding. In this case, the crystals were stable only in the presence of a high concentration of polyethylene glycol in the suspending medium or, alternatively, in the presence of a high proportion of methaemoglobin. The role of methaemoglobin in stabilizing crystals of T-state haemoglobin is not surprising, since it is known that the transition from T to R is less favoured by the conversion of deoxyhaemoglobin to methaemoglobin than by the conversion of deoxyhaemoglobin to oxyhaemoglobin (Perutz *et al.* 1974, 1976) and, furthermore, that oxidation of haemoglobin does not cause any crystal damage (Liddington *et al.* 1988; Mozzarelli *et al.* 1991*a*).

The time course of oxygenation of stable T-state crystals is shown in figure 4. Oxygenation of these crystals is fully reversible. At low methaemoglobin (2%) and low polyethylene glycol concentration (36% by mass), the time course of oxygenation is complex (figure 4) and fine cracks appear on the crystal surface as full saturation is achieved. We interpret this behaviour as the result of a conformational change of the protein, possibly the T to R quaternary transition. Oxygen can be removed and we have evidence, from the change in the polarization ratio, that haemoglobin tends to return to the quaternary conformation of the deoxygenated molecule. We are now looking for conditions to minimize crystal cracking, with the hope of being able to monitor, via the spectral changes associated with oxygenation, the allosteric transition of haemoglobin while it occurs as a collective process within nearly intact crystals.

5. Discussion

The electronic spectra of chromophores tightly packed within a protein matrix are sensitive probes of their local environment. In most cases, they appear to be

† 1 Torr ≈ 133.3 Pa

unaffected by the intermolecular interactions supporting crystallization. However, in other cases, spectrochemical probes have reported local structural differences between the solution and the crystal state of the protein (Johansen & Vallee 1971, 1973).

A more stringent criterion for comparing the structure of protein active sites in the two physical states is the correlation between a characteristic electronic spectrum and function. We have initially validated this criterion by showing that the spectrum of indoleacryloyl-α-chymotrypsin in the crystal, identical to that of the catalytically active acyl-enzyme in solution, is diagnostic of the same, pH-dependent, reactivity of the acyl bond (Rossi & Bernhard 1970). A minor lattice-induced alteration of the active site of γ-chymotrypsin is evidenced by the shift of the rate of deacylation-pH profile (Merli & Rossi 1986). On the basis of solution studies, Rossi & Bernhard (1971) concluded that a conformational change of indoleacryloyl-chymotrypsin is intimately associated with the time course of catalysed deacylation. It would be of interest to verify by time-resolved crystallography that, in the crystal, this structural change occurs with the pH-dependent rate of acyl bond hydrolysis rather than as a kinetically distinct step.

Different absorption spectra of crystalline β-2-furylacryloyl-glyceraldehyde-3-phosphate dehydrogenase, in the absence and in the presence of saturating concentrations of NAD^+, have been shown to be associated, respectively, with the inert and the catalytically active states of the acyl-enzyme (Berni *et al.* 1977; Vas *et al.* 1979), as in solution (Malhotra & Bernhard 1968, 1973). Furthermore, activation of the acyl bond by NAD^+ appeared to be mediated by a protein isomerization (Mozzarelli *et al.* 1982). It would be extremely interesting to compare the protein conformations as well as the acyl bond configurations of the inert and NAD^+-activated acyl-enzyme to explain their different electronic spectra and reactivity.

Negative cooperativity, or half-of-the-sites reactivity, of D-glyceraldehyde-3-phosphate dehydrogenase in the acylation reaction was found to have the same kinetic basis in the crystal and in solution (Vas *et al.* 1979). Its structural basis, which has been attributed either to a pre-existing or ligand-induced asymmetry among the sites, remains to be investigated and might be elucidated by a time-resolved structural study on the timescale of minutes.

It should be noted that the reactivity of the active site cysteines towards β-2-furylacryloylphosphate is most likely associated with very specific and subtle properties of the active site. This is evidenced by the absolute lack of reactivity of the *Bacillus stearothermophilus* enzyme, both in the crystal and in solution (A. Mozzarelli, unpublished results). The three-dimensional structure of this enzyme has been determined (Biesecker *et al.* 1977) and the active site geometry appears to be very similar to that of the muscle enzyme (Moras *et al.* 1975). Therefore, the intriguing structural basis of negative cooperativity of the muscle enzyme might turn out to be hard to detect.

Larger conformational changes appear to be associated with acylation of all four sites of the enzyme by the natural substrate 1,3-bisphosphoglycerate, since this reaction was found to lead to cracking of the crystals of the *Palinurus vulgaris* enzyme. In the case of the *Bacillus stearothermophilus* enzyme, the acylation reaction was very slow, probably because of strong lattice interactions that prevent the conformational transition.

In the case of crystalline aspartate aminotransferase from pig heart mitochondria,

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the coenzyme rotation predicted by Karpeisky & Ivanov (1966) and the conformational changes associated with transamination (Arnone *et al.* 1985; Jansonius *et al.* 1985) appear to be compatible with the crystal lattice. In fact, we determined almost the same equilibrium dissociation constants and kinetic constants holding for the corresponding reactions in solution. Unfortunately, the enzyme crystals we have used have not been studied by X-ray crystallography. Both the mitochondrial isoenzyme from chicken heart (Kirsten *et al.* 1983) and the cytosolic isoenzyme from pig heart (Metzler *et al.* 1988), which have been thoroughly investigated, present a lattice induced asymmetry between the catalytic sites.

In crystals of tryptophan synthase exposed to L-serine, the equilibrium distribution of β-subunit-bound catalytic intermediates does not correspond to that observed in solution. In particular, the pH dependence of the interconversion between external aldimine and α-aminoacrylate Schiff bases appears to be controlled by the ionization of one rather than two ionizing groups. Under some conditions the titration shown in figure 1*d* may not be fully reversible. However, the allosteric effects exerted by α-subunit ligands on the β reaction are not prevented by lattice forces and allow to modulate the distribution of intermediates (Mozzarelli *et al.* 1989).

Since interconverting species can be obtained within the same crystal, and their relative concentration is controlled by pH and α-subunit ligands, it becomes possible to plan time-resolved measurements of reactions initiated by a pH-jump or liberation of a caged α-subunit ligand. Preliminary experiments of this kind are underway in our laboratory.

The lattice constraints that prevent the T to R quaternary transition of haemoglobin have been exploited to investigate functional properties of a T-state molecule in the same environment where its structure is defined.

The nearly complete saturation of reduced haemoglobin at high oxygen pressures, which we observe unambiguously, is at variance with the conclusion, based on crystallographic results, that oxygen is bound only to the α subunits. Since the α and β subunits exhibit somewhat different oxygen affinities, one would expect the Hill coefficient to be lower than one. It is conceivable that the observed n value, very close to one, results from the interplay of site asymmetry and moderate positive cooperativity. A distinctive feature of the crystalline protein is that, in the presence of intact salt bridges, oxygen binding is pH-independent. On the contrary, the K_1 for binding of the first oxygen molecule in solution appears to be pH-dependent (Lee *et al.* 1988).

The p50 of crystalline T-state haemoglobin is about seven times higher than that measured in solution for the binding of the first oxygen to a T-state molecule (Imai 1982). It is interesting to note that a similarly low affinity has been observed in solution in the presence of clofibrate derivatives (Lalezari *et al.* 1990). Maybe lattice interactions stabilize a protein conformation which is the same or very similar to that stabilized in solution by strong allosteric effectors. However, haemoglobin Rothschild (37β Trp $\rightarrow$ Arg), crystallized from polyethylene glycol solutions as haemoglobin A and in a structurally similar T-state, exhibits a p50 as low as 20 Torr, at 20 °C (A. Mozzarelli, unpublished results).

6. Conclusion

Lattice interactions limit the conformational flexibility of proteins and therefore might prevent full expression of their binding and catalytic activity.

However, within the frame of the crystalline environment, it has been possible to observe numerous function-related phenomena and, in a few cases, to measure their time course by single crystal microspectrophotometry.

A completely new area of investigation includes physiological reactions that are expected to occur on the timescale of milliseconds or less and, therefore, cannot be adequately initiated by diffusion of reagents.

An approach to the study of reactions involving natural substrates has been to cool the crystals to subzero temperatures (Petsko 1975; Makinen & Fink 1977; Fink & Petsko 1981; Douzou & Petsko 1984; Hope 1990). Enzyme–substrate intermediates can thus be trapped in considerable amount for both spectroscopic and structural studies and, moreover, the time course of their accumulation and further transformations can be measured.

An alternative promising approach is photoactivation of stable precursors of substrates and other reagents, so called 'caged compounds' (Kaplan *et al.* 1979; McCray & Trentham 1989), pre-accumulated within the crystal channels, or photoactivation of a caged enzyme (Stoddard *et al.* 1990). The initiation of reaction synchronously and uniformly on very short times is the major goal to achieve in view of the time-resolved crystallographic experiments made feasible by the decrease of X-ray exposure time through the use of a synchrotron X-ray source and the Laue diffraction techniques (Moffat *et al.* 1984; Helliwell 1984; Cruickshank *et al.* 1987; Hajdu *et al.* 1988; Moffat 1989; Hajdu & Johnson 1990).

The possibility of matching the timescale of chemical and structural events not only by optical methods but also by time-resolved X-ray crystallography opens perspectives absolutely undreamed of at the time the kind of microspectrophotometric measurements here reported were first designed to observe time-dependent events within single protein crystals.

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Discussion

G. Petsko (*Brandeis University, Massachusetts, U.S.A.*). Those of us who do time-resolved crystallographic experiments are sensitive to the importance of carrying out

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microspectrophotometry experiments on our specimens first, but apparatus for such work is not generally available. Please comment on the difficulties and cost of constructing such devices.

G. L. Rossi. A microspectrophotometer must meet the standards of a very high-quality spectrophotometer in a wide wavelength range, possibly including ultraviolet and near infrared. High-quality microspectrophotometric equipment, built to meet the requirements of accurate polarized absorption measurements, is commercially available, at quite high cost. Home-made instruments can be designed to satisfy specific requirements, as handling of samples at subzero temperatures or the collection of spectroscopic data on line with the X-ray crystallographic apparatus. Obviously, costs will vary enormously depending on the performances required, the skill of the shop, etc. The general principles of design of a microspectrophotometer have been published and discussed, in particular in connection with potential errors in polarized absorption measurements due to the use of high numerical aperture objectives (Hofrichter & Eaton 1976 and references therein; Churg & Makinen 1978; Hofrichter 1979; Zelano *et al.* 1985).

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