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Cryoradiolysis and cryospectroscopy for studies of heme-oxygen intermediates in cytochromes P450

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Abstract

Cryogenic radiolytic reduction is one of the most simple and convenient methods of generation and stabilization of reactive iron-oxygen intermediates for mechanistic studies in chemistry and biochemistry. The method is based on one-electron reduction of the precursor complex in frozen solution via exposure to the ionizing radiation at cryogenic temperatures. Such approach allows for accumulation of the fleeting reactive complexes which otherwise could not be generated at sufficient amount for structural and mechanistic studies. Application of this method allowed for characterizing of peroxoferric and hydroperoxo-ferric intermediates, which are common for the oxygen activation mechanism in cytochromes P450, heme oxygenases and nitric oxide synthases, as well as for the peroxide metabolism by peroxidases and catalases.

1. Introduction

The detailed understanding of the chemical mechanism implies the knowledge of the most important intermediates of the reaction, their structure and properties. This information can be experimentally obtained by means of the fast kinetic methods, by slowing down the progress of reaction by varying experimental conditions, by trapping of intermediates, or using combination of all these methods. Usually significant results can be achieved by using low temperatures, just because rates of almost all chemical reactions strongly depend on temperature. Thus, data collection at low temperatures can often provide more details on chemical kinetics and on the properties of intermediates at the same experimentally available time range.

This review centers on the application of optical spectroscopy to studies of unstable iron-oxygen intermediates in heme enzymes in frozen glassy solutions. Systematic use of cryogenic temperatures is also essential in many other applications of spectroscopic methods in biochemistry and biophysics. A major part of EPR and Mössbauer data is collected at cryogenic temperatures. Other examples of cryogenic spectroscopic methods include most of the X-ray spectroscopic methods such as XAS, EXAFS, etc., and temperature-dependent magnetic circular dichroism, which provides indispensable information on the EPR-silent systems (1,2). Moreover, most of X-ray crystallographic data on proteins are also obtained at 100 K to minimize radiation damage caused by the modern bright synchrotron sources. Thus, understanding the general aspects of protein behavior at low temperatures is important, including dealing with possible effects of the frozen aqueous or mixed aqueous – organic solutions on the physical and chemical properties of proteins.

Historically cryogenic optical spectroscopy was used in heme protein studies as long as in 1920, reviewed by Keilin and Hartree (3). More recent applications include resolution of vibrational structure and conformational assignment of circular dichroism spectra of aromatic amino acids (4,5), MCD studies of heme enzymes (6,7) nonheme metalloproteins (8) and comprehensive characterization of photocycle in bacteriorhodopsin (9,10). Various experimental methods have been developed to study protein dynamics and relaxation using cryogenic optical spectroscopy, such as hole burning spectroscopy (11), photolysis and recombination of CO, NO and O₂ in heme proteins (12-15), and deconvolution of temperature-dependent absorption spectra (16). Optical spectroscopy is also used to monitor the redox state of metalloproteins directly during data collection in cryogenic X-ray crystallographic experiments (17,18).

1.1 Solvents for low-temperature and cryogenic spectroscopy

The classic book by Meyer (19) provides a comprehensive and detailed description of application of cryogenic techniques for optical spectroscopy, including different examples of design of low-temperature cells and cryostats, as well as an indispensable collection of physical and chemical properties of mixed cryosolvents which are used at cryogenic temperatures. Several other books (20,21) and (22) contain a thorough analysis of fundamental theoretical and experimental aspects of chemistry and biochemistry at low temperatures, particularly in the frozen solutions. Many review articles (23-27) provide an excellent introduction into the field of cryobiochemistry and cryoenzymology and offer both general theoretical concepts and valuable experimental information.

For many spectroscopic methods such as EPR, NMR, XAS, Mössbauer, the homogeneity of the frozen solution is not an essential condition and microcrystalline or powder samples can be studied with no experimental difficulties. This is not the case for the optical absorption spectroscopy, where light scattering in the highly turbid samples can completely obscure absorption spectra even in thin layer cells, especially in the UV and visible range, 200 – 800 nm. Thus, it is important to use solvents which form optically transparent clear glass when frozen at low temperatures. Many organic and inorganic solvents, such as methylcyclopentane, isooctane, ethanol, isopropanol, glycerol, triethanolamine, boric, phosphoric, and sulfuric acid, can form glasses transparent at 77 K. In addition, binary or ternary solvent mixtures are often used in cryospectroscopy because they may form glasses when cooled, even despite their constituents crystallize with phase separation of pure solvent and freeze concentrated solute. A table of optically clear glassy solvents is given in (19), Chapter 8. However, the choice of solvents for biopolymers is usually limited to mixtures of water with selected alcohols (28,29), concentrated solutions of sugars (30,31) or salts (23), because most common organic solvents unfold or precipitate proteins and inactivate enzymes. For proteins the most popular cryosolvents are binary mixtures of water with ethylene glycol or glycerol, although methanol (32,33), dimethyl sulfoxide, and dimethylformamide (32), as well as ternary mixtures of water with combinations of these solvents (29) also can be used. To prevent crystallization of water upon freezing, 50 – 70% w/w fractions of organic solvent are used in cryospectroscopic studies. For instance, FTIR study of water-glycerol mixtures at low temperatures indicated that phase separation and crystallization of water is fully suppressed at 65 % w/w (60% v/v) fraction of glycerol (34).

Fortunately, in most cases enzymes remain folded and active in such mixed cryosolvents even at extremely low temperatures (32,33,35-38) despite the very well-known phenomenon of 'cold denaturation' (39,40).

An important aspect of biochemical studies at low temperatures is the necessity to control the pH of aqueous – organic solutions and to avoid large shifts of pH while changing temperature. The methods of pH measurements and the properties of the most popular buffer systems in mixtures of water with alcohols at subzero temperatures have been studied and reviewed by Douzou and colleagues (29,36,41). Despite the fact that some buffers, such as acetate and phosphate have much smaller pK_a temperature dependence than amine buffers, a general recommendation for the most stable buffer system is difficult because of the changes of ionization enthalpies of these groups in organic and mixed solvents. Several reviews by Douzou provide tabulated reference data for several buffers in mixed aqueous – organic solvents (24, 29, 36).

Another source of large pH changes at low temperature is phase separation and partial crystallization of water in frozen solvents. Orii have shown that pH may drastically change in the most commonly used buffer systems upon freezing of the solutions (42). For instance, pH in phosphate buffer solutions could drop by 2 - 3 units during freezing (43). This effect was correctly attributed to the sharp increase of the buffering salt concentration along with the progress of the sample freezing and formation of the grains of pure aqueous ice microcrystals (42,43). The same phenomenon was described in an optical spectroscopic study of protonation behavior of the dye Cresol Red in the frozen aqueous solutions in the presence of organic and inorganic acids and bases (44). The drastic change of pH during freezing of aqueous solutions represents a very important aspect in cryogenic storage of biochemical samples, and the presence of 10 – 15% glycerol or other cryoprotectant is important to prevent the damage which may be caused by such changes. The systematic study of pH changes in acetate, phosphate and cacodylate buffer solutions as a function of pH was published by Jung and coauthors (45). They described in detail an indirect method of pH measurements below 0° C using several acid-base indicator dyes. In general, an increase of pH by 0.6 – 1.1 units has been detected at low temperatures. To avoid such changes, a new method was developed recently (46), which allows to create buffers with temperature-independent pH by mixing of two buffers, one with positive and another with negative temperature coefficients of their pH. This approach can be very useful if constant pH has to be maintained in the broad temperature range.

1.2 Temperature dependence of chemical kinetics

For many chemical reactions, the dependence of their rates on the temperature obeys the Arrhenius equation:

$$k=A \exp (-E_a/RT)$$

This equation holds for the reactions with the strong temperature coefficient and hence relatively high activation energies, i.e. much higher than RT (0.6 kcal/mol at T=300 K). For such reactions, the Arrhenius plots are linear in the accessible temperature intervals, and

activation energy E_a , which is approximately equal to the enthalpy of activation (47), can be estimated from the slope of $\ln(k)$ v. $1/T$.

As an example, consider stability of oxy-ferrous complex in cytochromes P450, or any other heme protein against autoxidation at different temperatures. Concentration of oxy-complex is determined by the rates of oxygen binding to the ferrous heme protein and of oxidative decomposition of this complex with dissociation of superoxide anion: For many heme proteins, experimentally observed apparent rates of oxygen binding are in the range $10^2 - 10^3 \text{ s}^{-1}$ at ambient oxygen concentrations. Thus, oxygen binding is fast at air-saturated buffers and usually does not limit complete oxygenation of the reduced heme proteins. However, for some cytochromes P450 as well as for NOS, the measured apparent autoxidation rates are also very high, and may reach the same order of magnitude, up to 10^2 s^{-1} and higher (48,49). For these enzymes, the maximum concentration of oxy-ferrous complex, which can be reached by fast mixing of the reduced protein solution with aerated or oxygen saturated buffer, is determined by the ratio of the rates of oxygen binding and oxidative decomposition of oxyferrous complex, as shown in Figure 1. To increase the population of the oxy-complex, it is necessary to maximize this ratio by the appropriate choice of the experimental conditions, as shown in the Figure 1a. Acceleration of oxygen binding is limited by oxygen solubility and cannot be significantly improved without using high-pressure technique (see (50) for an example). However, the ratio $k_{\text{binding}}/k_{\text{autox}}$ may be varied significantly based on the substantially different temperature dependence of these rates.

Autoxidation reactions have been shown to have high activation energies in cytochromes P450 and other heme proteins, typically in the range 15 – 25 kcal/mol (48). For example, the Arrhenius activation energy for autoxidation of CYP3A4 saturated with the substrate testosterone is 22 kcal/mol (48), and for autoxidation of CYP19 with androstenedione, it is 18 kcal/mol (51). Contrary, the O_2 and CO binding rates have significantly lower activation energies in the range 6 – 9 kcal/mol, as documented for several heme proteins (52-55). Hence, the ratio $k_{\text{binding}}/k_{\text{autox}}$ increases with decreasing temperature. The resulting increase in stability of oxygenated intermediate is illustrated in Figure 1b, where the same time dependences of the fraction of oxy-ferrous complex are calculated at different temperatures. In addition, the characteristic time window available for the measurements and other manipulations with unstable oxygenated heme enzyme is significantly increased at low temperature. Hence, working at low temperatures provides new opportunities for mechanistic studies in chemistry and biochemistry, as reviewed earlier (20,22,56) .

1.3 Cryogenic radiolytic reduction

Radiolysis is decomposition or fragmentation of the molecule by high-energy particles or photons commonly termed ionizing radiation. Most popular and convenient to use are photons in the X-ray or gamma-range, or electrons from the linear accelerators. Sometimes radioactive isotopes such as ^3H , ^{32}P , ^{35}S , also can serve as internal source of high-energy electrons (57-59). The yield of radiolysis is usually measured as an average number of corresponding products per 100 eV absorbed by the sample. The main products of the water radiolysis are hydroxyl radicals, hydrated electrons, hydrogen atoms, and several other

highly reactive intermediate species which quickly react with solutes or recombine to produce more stable compounds such as hydrogen peroxide. Radiolysis of alcohols, which include ethylene glycol and glycerol, results in the formation of hydrogen atoms, CO, aldehydes, ketones, alkanes and alkenes. In all cases, the final radiolytic yield depends on the secondary reactions between primary radicals and on the presence of other compounds (60,61). However, at cryogenic temperatures almost all reactive products of radiolysis are immobilized in the frozen solvent matrix, or trapped by radical quenchers. Only solvated electrons are mobile enough to escape spurs and to react with the heme protein complexes even in frozen solutions at 77 K. .

A large number of published works utilized γ -irradiation from ^{60}Co source for cryoradiolytic reduction of cytochromes P450 and other heme proteins (59,62,63). Photons from this source, with energies 1.13 and 1.3 MeV, interact with atoms with low Z (atomic mass) mostly through inelastic Compton scattering with emission of lower energy photon, and generation of free electron and cation radical as primary products of this scattering (60). The scattered photon and electron generate more electrons with the energy of the initial γ -photon gradually dissipated in the cascade of scattering events. According to the experimentally measured yields (60), one γ -photon with the energy 1 MeV absorbed in water will generate approximately 27,000 electrons, which means that approximately 2 mM average concentration of free electrons will be generated by 1 Mrad absorbed dose from ^{60}Co -source (1 rad is defined as $6.24 \cdot 10^{13}$ eV/g, or 10^{-5} J/g of absorbed energy). The actual concentration of generated electrons in ice at 77 K may be several times lower because of recombination of electrons with parent radicals, and the typical doses used in cryoradiolytic reduction range from 2 to 10 Mrad. Recombination of electrons with radicals also results in a saturation behavior of cryoradiolytic reduction with increasing dose, and the yield for metalloproteins usually is not improved by prolonged irradiation with the doses higher than 4 – 6 Mrad dose (63,64).

Recently, the method of cryoradiolytic reduction was established as an indispensable tool in the studies of unstable iron-oxygen intermediates in cytochromes P450 and other heme enzymes (59,62,65-72). In these works the oxy-ferrous complexes of heme proteins have been prepared by direct oxygenation of the reduced ferrous protein and then frozen in liquid nitrogen. One-electron reduction of these samples by radiolytic electrons generated using irradiation with high energy photons at 77 K (X-rays or γ -rays) results in the formation of peroxo-ferric or hydroperoxo-ferric complexes. These fleeting intermediates of heme enzyme catalysis could not be isolated in ambient conditions, despite numerous attempts. However, being immersed in liquid nitrogen, they are stable for many months and can be studied using various spectroscopic methods, such as EPR (62,65,67,73-76), UV-Vis absorption spectroscopy (58,59,69,70,76-79), resonance Raman (69,71,72,79,80), Mössbauer (67), and EXAFS (81).

2. Materials

1. Methacrylate UV-vis enhanced semimicro cells for optical absorption (Fisher Scientific, Cat 14-955-128). Polystyrene semimicro cells can be also used at wavelengths longer than 320 nm (Cat 14-955-127).

2. NMR (5 mm outer diameter) or EPR (4 mm outer diameter) tubes, glass or quartz, for resonance Raman or EPR samples. For EPR, quartz is preferred for higher purity and much lower contamination with iron.
3. Glycerol, ultrahigh purity (Fluka), dithionite (Sigma), phosphate buffer for appropriate pH.
4. Dewar flasks, including one for cryogenic irradiation, 350 ml, outer diameter 8.5 cm, height 15 cm to fit the irradiation compartment of Gammacell-220 275 ⁶⁰Co source, which has the shape of cylinder with 20 cm height and 13.5 cm diameter.
5. Dry ice and ethanol for two cold baths (see Subheading 3.1, step 3).
6. Long thongs for sample manipulation when fully immersed in liquid nitrogen.
7. Protective eye goggles.
8. Large storage Dewar for prolonged storage of samples at 77 K fully immersed in liquid nitrogen.
9. Equipment for the cryospectroscopy. (1) Commercially available optical cryostat or home-made, as described (our papers). (2) Thermocouple and thermometer for the temperature control in the broad range, from 77 to 300 K (Fisher). (3) Liquid nitrogen to use as a cooling agent. Warning: work with liquid gases has to be performed according to the safety instructions!
10. Spectrophotometer with a large enough sample compartment to accommodate the optical cryostat. Sample compartment has to be purged by a constant flow of dry air or nitrogen to prevent condensation of atmospheric water vapor on the cryostat to avoid light scattering during spectroscopic measurements.
11. Access to the large ⁶⁰Co source or alternative source of ionizing radiation.

3. Methods

3.1 Preparation of oxy-ferrous complex in cytochromes P450

1. Solutions of cytochromes P450 are deoxygenated for several minutes under flow of humidified nitrogen or argon of high purity and transferred to the anaerobic chamber. Deoxygenation of glycerol (ultra-high purity glycerol is used to minimize contamination with organic peroxides) is achieved by multiple freeze – pump – thaw cycles under pure nitrogen or argon.
2. After addition of glycerol (15% v/v), cytochrome P450 is reduced by two-fold molar excess of dithionite, added from the freshly prepared solution at the same phosphate buffer. The reduced sample is loaded into the gas tight Hamilton syringe and kept in the anaerobic chamber till oxygenation.
3. To provide the maximum stability of the oxy-ferrous complex, two cold baths with different ratios of ethanol/dry ice have been used, Bath A with excess ethanol for -30° C, and Bath B with excess of dry ice at -70° C. Note that temperature of Bath

A is important for the stability of oxy-complex, and has to be maintained at the desired level by gradual addition of dry ice.

4. The methacrylate cell (Fisher – cat No.), or the 4 mm EPR tube (WILMAD, cat no.) with 70% glycerol/buffer saturated with oxygen gas is equilibrated at -30° C by immersion into the first bath.
5. The concentrated solution of the reduced cytochrome P450 is then quickly inserted into this glycerol/buffer using gas tight syringe and stirred for 25 – 30 seconds using a cold metal stirrer. As soon as the sample looks homogeneous, it is transferred to the second bath at -70° C to quench the oxy-ferrous complex.
6. The good thermal contact of the cell with the sample and the bath provides fast cooling rate and ensures longer life-time of oxyferrous complex. After cooling at -70° C for two minutes, the cell is quickly transferred to the optical cryostat which is precooled at for -100° C, or 170 K. The sample is gradually cooled down from this temperature to 77 K, and the absorption spectra are taken at several intermediate temperatures to ensure the formation and stability of oxy-ferrous complex.

After cooling to cryogenic temperatures, the solvent solidifies and forms optically transparent glass, where diffusion of even smallest molecules is severely impaired, and no autoxidation is observed at the temperatures below 200 K. Stabilization of oxy-ferrous intermediates in cytochromes P450 at cryogenic temperatures makes possible application of slow spectroscopic and structural methods for comprehensive studies of both oxyferrous and peroxo-ferric complexes, which can be obtained by cryoradiolytic reduction.

Another approach can be used if oxygen isotope ^{18}O have to be used for vibrational spectroscopy (69,71,72,79). In such case, aerobic oxygenation has to be replaced by application of isotope-enriched oxygen using gas tight syringe and inserting the needle through the septum or on the Schlenk line. Deoxygenation of concentrated P450 solution and ultra-pure glycerol is accomplished as described above. Next, they are mixed in appropriate proportions in the anaerobic chamber and ferric cytochrome P450 is reduced by addition of small molar excess of dithionite solution of known concentration. To maintain anaerobic conditions, the samples in NMR or EPR tubes with rubber septa are packed into the second closed container and only after that transferred from anaerobic chamber into the -80° refrigerator. After cooling the samples can be safely connected to the Schlenk line and purged with nitrogen or argon before application of isotope-enriched oxygen $^{18}\text{O}_2$ or other gas.

3.2 Cryogenic radiolytic reduction

1. Samples are placed into the Dewar flask filled with liquid nitrogen and covered with a styrofoam stopper to minimize evaporation of the coolant during irradiation. The Dewar flask has to be made of glass, possibly silvered, but not stainless steel or other metal, to avoid strong absorption of γ -rays. The styrofoam cover can be easily cut from the styrofoam boxes to match the size of the Dewar flask and to allow holes for the NMR tubes, if necessary.

2. The Dewar containing samples and filled with liquid nitrogen is placed into the irradiation compartment of ^{60}Co source (Gammacell 220 has a cylindrical irradiation compartment with approximately 23 cm height and 16 cm diameter). Liquid nitrogen constantly evaporates during irradiation and has to be added every 90 – 100 minutes.
3. After irradiation all samples contain high concentrations of trapped electrons, which have strong absorption band in the visible region. Thus, the samples for optical absorption spectroscopy before measurements have to be illuminated for 10 – 15 minutes with the visible light ($\lambda > 450 \text{ nm}$) from the Xe lamp or some analogue, to photobleach these electrons and to make the samples transparent. During photobleaching samples are fully immersed in liquid nitrogen.

4. Notes

Making oxy-complex in aromatase and CYP3A4 by quick mixing of the concentrated anaerobic solutions of reduced P450 in 15% glycerol with 6-fold volume excess of 70% glycerol/phosphate buffer saturated with oxygen and precooled at -30°C . This approach is preferable for the enzymes with very fast autoxidation rates, because oxygen binding happens at low temperature, where the oxy-ferrous complex has a longer half-life. Typical Arrhenius activation free energies for autoxidation of heme enzymes are in the range of 15 – 25 kcal/mol, corresponding to approximately 2.2 – 4.3 fold increase of the half-life of oxy-complex with every 10 degrees decrease of the temperature. This strong temperature dependence of autoxidation rate implies an increase of the half-life of unstable oxy-ferrous complexes in cytochromes P450 from tens of milliseconds at 37°C to hundreds of seconds at -30°C , Figure 2. We utilized this method to obtain oxycomplexes of CYP19 and CYP3A4 for the optical absorption and EPR studies of peroxo-intermediates obtained by radiolytic reduction of oxy-ferrous precursors (82).

Making unstable oxy-ferrous complexes - mixing at low temperatures in cryosolvents or bubbling cold gas though the anaerobic solution of reduced heme protein. Oxy-ferrous complex in cytochromes P450 is usually only marginally stable at ambient conditions, and irreversibly decomposes with the escape of superoxide from the remaining ferric hemoprotein with typical rates $0.01 - 10 \text{ s}^{-1}$ (48). However, high activation energies of autoxidation reactions, 15 – 24 kcal/mol, provide very steep increase of oxyferrous complex stability with the decrease of temperature, approximately by three orders of magnitude on cooling from the room temperature to -30°C , Figure 2. Thus, oxygenation of cytochromes P450 can be accomplished by manual mixing at -30°C in aqueous glycerol buffer.

4.1 Radiolytic reduction

Ionizing radiation, i.e. high-energy particles or photons such as X-rays or gamma-rays, interacts with matter non-selectively, generating electrons and radicals. Using electrons generated by radiolysis of the solvent radiation, one can initiate the desired chemical reactions, both in solution and in solid state. While in solution multiple reactive radicals generated by radiolysis can interfere with the process under study through side reactions, and the overall radiochemical effect is in most cases oxidative transformation of the solute.

Contrary, in the solid state the diffusion of all products of radiolysis with the exception of electrons is impaired, and side reactions of the target compound with non-specific radicals can be prevented by keeping the sample well below melting temperature. Radiolytic electrons, which escape recombination and lose their kinetic energy through inelastic scattering on the solvent atoms, are either trapped in potential wells formed by polar groups or react with redox centers. Such reaction include one-electron reduction of metal ions, i.e. from Fe^{3+} to Fe^{2+} , formation of hydrogen atoms from protons in solution, or formation of organic radicals (83).

4.2 Mechanism, yield, stability of cryoreduced intermediates

In cryoradiolytic reduction, the solvent serves as the primary source of electrons for the reduction of target compound. In this sense, cryogenic radiolysis is different from other approaches in matrix isolation chemistry, where reagents or reactive complex are perturbed directly, usually using UV-photolysis. The latter requires a source of specific excitation and inert matrix, which does not interact with irradiation used in the experiments. In turn, the method of cryoradiolysis uses nonspecific ionizing radiation, which may interact with the compound of interest as well as with the solvent matrix. For the dilute solutions of the reactive complexes, the volume fraction of the solvent is much higher, and the effect of direct radiolysis of the target compound can be neglected. Note that the solvent can also serve as a selective quencher of undesired radiolysis products. For example, glycerol or ethylene glycol efficiently trap and immobilize hydroxyl radicals in experiments on cryogenic radiolytic reduction of metalloproteins and greatly improve the yield of solvated electrons, which in turn results in higher yield of reduced protein (64).

Conclusion

Cryogenic stabilization in frozen glassy solvents is a very useful method for the studies of unstable iron – oxygen intermediates in heme enzymes. Combining this method with cryogenic radiolytic reduction allows for accumulation of one-electron reduced transient peroxo-ferric and hydroperoxo-ferric complexes and for detailed spectroscopic and structural characterization of such complexes, which usually cannot be isolated and stabilized by other methods.

Additional reading

Different aspects of low-temperature spectroscopy have been described in many useful reviews and original papers. Elaborated and systematic development of low-temperature methods for biochemistry by Pierre Douzou and his colleagues produced dozens seminal publications, summarized in many excellent reviews (24-26,35,84-87) and a book (20), which describe in detail theoretical basis and numerous indispensable details of experimental methods. The works of Roy Daniel and collaborators also provide a useful insight into the role of enzyme dynamics in the catalytic activity by using low-temperature methods and cryosolvents (27,28,88). Absorption spectra and MCD of unstable high-valent intermediates in heme enzymes have been described by Gasyna, Stillman and coworkers (76,89-91). Application of cryogenic optical spectroscopy to biophysical chemistry of proteins, including heme enzymes, has been described by Vanderkooi, Friedrich and

colleagues (11,34,92-98). Frauenfelder and colleagues systematically applied various spectroscopic methods at cryogenic temperatures to study fundamental aspects of protein structure and dynamics (12,99-101).

A number of analytical and preparative biophysical and biochemical methods other than spectroscopic, also can be modified to take advantage of improved sample stability at sub-zero temperatures. Application of aqueous – organic solvents for extending the available temperature range down to -25° - -100° C and appropriate experimental modifications for work at such low-temperatures have been described for isoelectric focusing and electrophoresis (102-104), potentiometry (41), size-exclusion and affinity chromatography (84,105,106), and direct assays of enzymatic catalysis (28,86,88,107).

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Scheme 1.

Figure 1.

Kinetics of formation and decay of oxyferrous complex in cytochrome P450 as described by Scheme 1. (A) Fractions of ferrous (1), oxy-ferrous (2), and ferric (3) enzyme at $T = 279$ K as a function of time. (B) Fractions of oxy-ferrous complex as a function of time at three different temperatures: (1) 300 K; (2) 279 K; (3) 243 K. Parameters used for calculations: Rates $k_1 = 500 \text{ s}^{-1}$ and $k_2 = 5 \text{ s}^{-1}$ at $T = 300$ K, corresponding activation enthalpies $H_1 = 10 \text{ kcal/mol}$; $H_2 = 20 \text{ kcal/mol}$.

Figure 2. Arrhenius plot of the temperature dependences of autoxidation rate constants for CYP3A4. Experimental data (symbols) and linear fits (lines) are shown for CYP3A4 saturated with testosterone (triangles) and bromocriptine (circles). For clarity, corresponding absolute temperatures are shown on top, and calculated half-life times for the oxy-complex are listed on the right.