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Thermal access to amplified chemical potential and the determination of equilibrium constants in protein solutions at subfreezing temperatures

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Abstract

During rapid cooling of ferric heme protein solutions containing fluoride, locally concentrated ligand cannot fully equilibrate with heme before the temperature drops below 200 K and into the range where energy is insufficient for exchange with iron-bound water. When temperature is then jumped above 200 K, exchange of fluoride for bound water is activated. Between 200 and 240 K, further fluoride complex formation takes place over several minutes; its extent is measured along the kinetic curve by reimmersing the sample into liquid nitrogen and taking EPR spectra. Kinetic curves for replacement of iron-bound water by fluoride in horse aquo-ferrimyoglobin and human aquo-ferrihemoglobin, and corresponding equilibrium constants have been obtained at temperatures between 200 and 240 K. The reaction rates are affected by sucrose. Results indicate that the kinetics of exchange of fluoride for heme-bound water at subfreezing temperatures is protein specific and not diffusion-controlled, and is not affected by the phase transition of ice which takes place at subfreezing temperature. Free energy changes accompanying these reactions are largely continuous as the systems pass from above to below freezing.

Keywords: Bound water; Electron paramagnetic resonance; Fluoride complex; Freezing effects; Heme proteins; Thermal activation

1. Introduction

There is a substantial and growing literature which deals with experimental studies of protein systems in frozen solvents at cryogenic temperatures. In this literature are reported investigations of enzyme reaction mechanisms [1,2], of photosynthetic systems [3–5] and of protein dynamics [6–8]. However, because almost all biological activity occurs in largely aqueous solutions well above their freezing temperature, one can question the degree of biological relevance of experiments carried out at cryogenic temperatures. In particular, the process of freezing proteins in aqueous solution can, more or less, disturb some of their properties and thereby affect the results of protein-dependent biological experiments at cryogenic temperatures [9]. In this paper we report measurements of the equilibrium binding affinity of fluoride anion for ferrimyoglobin and ferrihemoglobin

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above and below the freezing temperature of the aqueous buffer, enabling a quantitative evaluation of how the freezing process and cold temperatures affect protein–ligand interaction. These measurements make use of a recently discovered effect and demonstrate a new technique, measurement of the kinetics of temperature-jump initiated changes in ligand binding at metal sites of proteins in frozen aqueous solution.

Investigation of the influence of the freezing process upon the binding of fluoride to ferric myoglobin and hemoglobin [10] led to a method for estimating the ligand concentration, greatly increased by freezing, in the neighborhood of the protein molecules. (Use of the term 'concentration' here is not precise; the solute-rich, icy, submicroscopic regions containing fluoride are not ideal solutions, and the fluoride chemical potential is unlikely to be strictly proportional to fluoride concentration). On the basis of such 'solute pooling' in frozen aqueous solution, Miller and McMillin [11] explain the observation of Severns and McMillin [12] that incubation at −20°C with two equivalents of fluoride almost quantitatively converts the T1Hg derivative of laccase to the difluoride adduct.

The discovery of thermally-activated replacement of water by fluoride, together with recognition of the slowness with which it proceeded, suggested a line of kinetic and equilibrium measurements to acquire new knowledge about this ligand exchange and permit comparison of room temperature and low temperature data. Here we determine dissociation constants of fluoride complexes at various temperatures between 200 and 250 K with a temperature jump technique. A sample containing heme protein and fluoride, frozen at 77 K in a non-equilibrium state, is brought to a temperature above 200 K; the dissociation constant at the temperature after the jump is then evaluated from the time course that leads to the equilibrium state. The long reaction times (minutes or longer) of protein systems at cryogenic temperatures makes monitoring by EPR and other physical methods practical. However, because of (1) phase transitions in ice, and (2) the possibility of diffusion limiting the rate of ligand arrival, one must be cautious in attributing kinetic effects observed after a (subfreezing) temperature jump to properties of the protein. These two problems were addressed in the experiments described below. Then, with experimental conditions of the frozen solution under firm control, the temperature jump technique was used. The thermodynamic and kinetic parameters so obtained at subfreezing temperatures are compared, by means of extrapolations from one temperature range to the other, with parameters obtained from liquid phase measurements above freezing, and the effect of the freezing process upon the protein–ligand interaction is discussed.

2. Materials and experimental methods

Horse heart myoglobin was purchased from Sigma (St. Louis, MO). Human hemoglobin was obtained from red blood cells. Ferrimyoglobin and ferrihemoglobin were prepared as described previously [13]. The protein solutions were buffered with 0.033 M potassium and sodium (K:Na = 5:2) phosphate at pH 6.3 (22°C), and centrifuged before use to remove suspended particles. The spectrophotometric purity value, defined as

$$PV = A_{\text{Soret peak}}/A_{\text{peak at 276–280 nm}},$$

was 5.6 for the ferrimyoglobin preparation and 5.7 for the ferrihemoglobin preparation.

Freezing and annealing of the protein solution in an EPR sample cell were monitored optically with the cell in a Varian variable temperature controller (VTC), as described previously [10]. EPR sample cells were made of polyethylene tubing, 2.6 mm ID, 0.4 mm wall, volume 0.048 ml. As before, the temperature of the sample solution was measured with a small diameter (0.13 mm) copper–constantan thermocouple in the cell. All EPR measurements were made at 77 K with a Varian 4500 X-band spectrometer modified in its microwave bridge; the sample cell was immersed in liquid nitrogen in a quartz/pyrex dewar, the quartz tail of which was inserted into a Varian 4531 general purpose cavity.

At 250 K, local equilibria between heme sites and fluoride at the surface of protein molecules take place efficiently while fluoride in the bulk frozen solution remains immobile. Above 250 K, the bulk solution begins to melt at elevated sucrose concentrations. These two factors make 250 K a generally useful annealing temperature, with temperatures

slightly lower still effective, and temperatures above to be avoided. In this paper 250 K is used as a standard and reference annealing temperature. The results of annealing at other temperatures are compared with those at 250 K.

3. Results

3.1. Temperature-jump experiments

The temperature-jump experimental procedure is depicted in Fig. 1A. A polyethylene EPR sample cell containing ferrihemoglobin and KF in phosphate buffer was immersed, at time A, in liquid nitrogen until the temperature of the sample reached equilibrium. At time B the cell was quickly moved from the liquid nitrogen to the dewar of a Varian variable temperature controller (VTC) where the temperature for 'annealing', T_a, was maintained by a stream of cold nitrogen gas for five minutes (period B to C). At time C the cell was moved from the VTC back into liquid nitrogen so that the EPR spectrum of the frozen solution in the sample cell could be measured at 77 K. Fig. 1B shows the EPR signal amplitudes of the low field peak as a function of the annealing temperature T_a. For the solution with [KF] = 1.23 mM (at room temperature), Fig. 1C shows the mole fraction x_a of the ferrihemoglobin–fluoride complex present after annealing at T_a; x_a was calculated by the method of the previous paper [10].

Measured above freezing temperature in this laboratory, the enthalpy and entropy of dissociation of the ferrihemoglobin–fluoride complex in 33 mM PO_4, pH 6.3 buffer, are 1.86 kcal/mol (7.79 kJ/mol) and −3.3 cal/(mol K) (−14 J/(mol K)), respectively. (Anusiem et al. [14] found the same enthalpy, but a somewhat different entropy, −2.6 cal/(mol K)). For these thermodynamic parameters, the equilibrium mole fraction of complex present in liquid solution just above freezing temperature is about 0.17 when the KF concentration is 1.23 mM (as it is in Fig. 1C). As discussed by Yang and Brill [10], during the few (ca. 4) seconds it takes for the sample to freeze when immersed in liquid nitrogen, the protein molecules are exposed to concentrated fluoride because fluoride (along with other ions) is expelled from the growing ice crystallites. Conse-

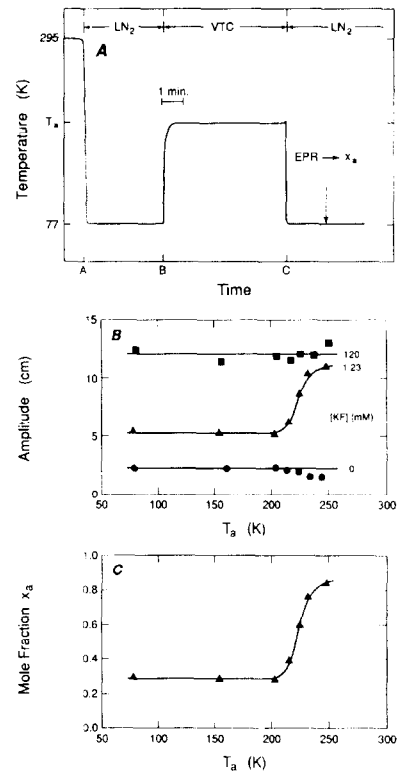

Fig. 1. Experiments with temperature jumps to annealing temperatures between 77 and 245 K. (A) The EPR cell, with sample solution in it, was immersed in liquid nitrogen at time A. In 6 seconds the temperature within the cell dropped below 100 K. At time B the cell was removed from the liquid nitrogen and quickly placed in the dewar of the Varian variable temperature controller (VTC) wherein nitrogen gas at temperature T_a was flowing at 850 l/h. The temperature T at the center of the cell was found to rise as an exponential function of time t, reaching the annealing temperature T_a according to

$$T = (T_a - 77)\cdot[1 - \exp(-0.17t)] + 77$$

where the temperature is Kelvin and the time is in seconds. After the 5 minute period B to C, the sample cell was removed from the VTC and immediately plunged into liquid nitrogen for measurement of the EPR spectrum (at 77 K). (B) The EPR signal amplitudes at the field corresponding to the $g \approx 6$ peak for the fluoride complex are plotted as a function of the annealing temperature T_a at 3 concentrations of potassium fluoride (in the homogeneous solutions prepared at room temperature): [KF] = 0 mM (●); [KF] = 1.23 mM (▲); [KF] = 120 mM (■). In all cases the sample solutions contained ferrihemoglobin, at a heme concentration of 0.245 mM, in 0.033 M, pH 6.3, phosphate buffer. The EPR instrument settings were: microwave frequency, 9.30 GHz; microwave power, 200 mW; field modulation frequency, 100 KHz; field modulation amplitude, 6 gauss. (C) For [KF] = 1.23 mM, the mole fraction $x_a(T_a)$ of the ferrihemoglobin–fluoride complex after annealing is plotted as a function of T_a.

quently, the average mole fraction of ferrihemoglobin–fluoride complex in the frozen solution is somewhat greater, i.e., in the range 0.22 to 0.28 (Fig. 1C, Fig. 3B(b), and Fig. 5C) before annealing, than the value, 0.17, applicable to equilibrium at 273 K. However, the system has been quenched in a state with the potential for reacting further. As can be seen from the results of annealing at temperatures between 77 and 250 K, this potential does not begin to be realized until the temperature is raised to 200 K. Below 200 K the signal amplitudes remain the same when the annealing time is extended to 40 minutes or longer. Above 200 K, the extent of formation of the fluoride complex increases rapidly with temperature, then levels off at about 250 K. It is clear that freezing produces a concentration of fluoride in a layer around the protein which is greater than that which can be calculated from the average mole fraction, 0.25, found prior to warming the sample. Below 200 K this fluoride is, apparently, constrained on the time scale of the freezing process from equilibrating with the nearby heme sites, but becomes available for binding when the temperature of the system is raised to 200 K or above. Alternatively, the water molecule which the fluoride ion replaces is constrained from leaving the heme below 200 K. There are many reports in the literature of phenomena requiring intraprotein and/or hydration water motions which become observable at about 200 K [15–28].

If, at all temperatures in the range 200–250 K, intraprotein motions explore the whole range of protein conformations which allow passage of water molecules out and fluoride ions in, then the mole fraction of fluoride complex would rise abruptly at 200 K, after which it would (if it behaved according to the thermodynamic parameters measured with solutions above freezing) decrease somewhat with increasing temperature. On the basis of measurements at 285 K and above, the extent of formation of the complex would be 41% at 200 K and 21% at 250 K. The results shown in Fig. 1C and Fig. 2B differ greatly from this behavior, and indicate that there is increased accessibility of conformations compatible with the exchange of fluoride for water as the temperature increases above 200 K.

The dissociation constant (K_d) of ferrihemoglobin fluoride was estimated to be 12 ± 1 mM at 250 K

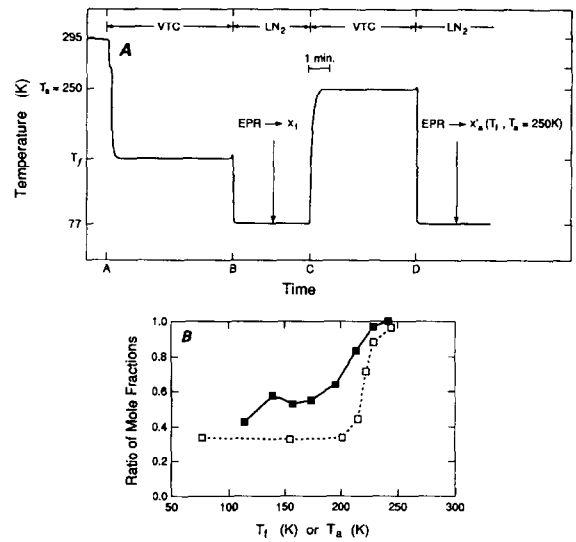

Fig. 2. Comparison of the extent of complex formation without annealing and with annealing at 250 K. (A) Protocol of Fig. 11a of Yang and Brill [10]. The sample solution in the EPR cell was placed in the dewar of the VTC for 6 minutes (time period A to B) with the bath of nitrogen gas at the temperature for freezing, T_f. At time B the cell was plunged into liquid nitrogen and the EPR spectrum taken to measure the mole fraction $x_f(T_f)$ of fluoride complex present after freezing in the gas at temperature T_f. At time C the cell was replaced in the VTC dewar where it remained for 5 minutes (C to D) with the bath of gas at 250 K; during this period of annealing, equilibration of the heme with fluoride surrounding the hemoglobin molecules took place. At time D the cell was, once again, plunged into liquid nitrogen to measure (with EPR) the mole fraction $x'_a(T_f, T_a = 250\ \mathrm{K})$ of the fluoride complex present after equilibration at annealing temperature $T_a = 250$ K, freezing having taken place at T_f. (B) -■-■- The information in Fig. 1b of [10] is replotted as the ratio of the extent (mole fraction x_f) of complex formation before annealing to that (mole fraction x'_a) after annealing at 250 K as a function of the temperature, T_f, of the bath of cold nitrogen gas in which the sample was frozen, i.e. $x_f(T_f)/x'_a(T_a = 250\ \mathrm{K})$ as a function of T_f. -□-□- The information in Fig. 1(C), where $T_f = 77$ K, is replotted as the ratio of the extent of complex formation after annealing at T_a to that at the standard annealing temperature, $T_a = 250$ K, i.e. $x_a(T_a)/x_a(250\ \mathrm{K})$ as a function of T_a.

[10]. With this value and that for the mole fraction at 250 K, 0.85, one calculates the average local concentration of fluoride around the protein molecules (in dilute solution) to be about 70 mM, although the average concentration of KF is only 1.23 mM. (It is such an enhancement of chemical potential, together with its availability above 200 K, that Miller and McMillin [11] effectively used to drive the formation

of the difluoride adduct of laccase). Thus the temperature-jumps in the experiments of Fig. 1 are accompanied by a concentration-jump in (accessible) ligand by a factor of about 55; this is equivalent to the fluoride occupying 2% of the original volume. If, after freezing, the protein molecules remain uniformly dispersed throughout the original volume, it is difficult to understand how the small volume containing the fluoride would be accessible to most of the protein molecules. One infers that the protein molecules are, at least somewhat, pushed during the freezing process into the small volume that the fluoride occupies after freezing and/or that the fluoride is concentrated in and around the hydration layer of the protein molecules.

3.2. The effects of 'phase' transitions during the freezing and annealing of protein solutions

When glassy water, which results from a non-equilibrium process and is in a metastable state, is warmed to room temperature, three transitions can be observed by calorimetric and optical measurements [29–31]. The first of these is the 'glass transition' from the vitreous state to that of a very viscous liquid; for pure water, the onset of the glass transition is at about 140 K. About 10 K warmer, ice crystals begin to grow at the 'crystallization' or 'devitrification' temperature. With additional warming there is, of course, the 'melting' temperature (273 K for pure water at 1 atm). Experimental evidence of the glass transition has been found only in measurements made upon glassy water with essentially no crystalline 'impurity' present.

For the experiments shown in Fig. 1 of this paper, frozen solutions were prepared by immersing polyethylene EPR sample cells in liquid nitrogen, thereby cooling at an average rate of 30 K s^{-1}. Ice crystals were present in the samples at 77 K, and no significant glass transition was expected to take place in the solvent phase of the frozen dilute aqueous solutions used for the temperature-jump experiments in Fig. 1. Water in the hydration layer around a protein forms a glass-like structure when the temperature drops below the freezing point of the aqueous solution [32]. Glass transition of the hydration water of myoglobin in crystals has been measured to occur in a broad range of temperatures, 180 to 270 K [20]. The hydration layer plays a very important role in protein dynamics and conformational stability [33]. Specific heat spectroscopy and flash photolysis experiments with 10 mM myoglobin in 75% (v/v) glycerol–water [8] suggest that solvent relaxation affects internal ligand binding, with a discontinuity at the temperature (about 200 K) where the rates of solvent relaxation and of processes in the protein are equal. When, below this temperature, the relaxation rate of the solvent is slow compared with that of the protein, the solvent and protein motions are decoupled. Thus a glass transition of the hydration water at the surface of a protein will influence protein reactivity and this influence is very likely to be reflected in kinetic measurements made at the transition temperature.

Under the conditions of the experiments reported here, the rate of freezing of sample solution following immersion of the polyethylene cell in liquid nitrogen was not sufficiently great to prevent crystallization altogether; rather, a hindered crystallization process took place and small, thermodynamically metastable ice crystals were formed in the solvent phase. In the course of annealing the frozen solution above the devitrification temperature, crystallization resumed and with it the conversion of the metastable system to states (still frozen) of lower free energy. Recrystallization will occur at various temperatures, depending upon the solute composition of the solution.

Qualitative evaluation of the importance of solvent transitions, of the kinds just discussed, on the replacement of water by fluoride is obtained by comparison of the results of experiments conducted according to the protocol of Fig. 11a of [10], shown again here in Fig. 2A, versus those presented in Fig. 1 above. Fig. 2B shows the information of Fig. 11b of [10] plotted as the ratio of the mole fractions before and after annealing (■). In these experiments for Fig. 2B, the temperature of the cold nitrogen gas bath for freezing the sample (called T_{bath} in the earlier paper, but here called T_f to distinguish it from the annealing bath temperature T_a), was varied while the annealing temperature was fixed at 250 K. Conversely, in the experiments of Fig. 1, T_f is fixed (the bath is liquid nitrogen) and T_a is varied. For Fig. 2B, the information in Fig. 1C is replotted as the ratio of the extent of complex formation after annealing at T_a

to the extent (mole fraction 0.85) after annealing at 250 K (□). While in both kinds of experiment the annealing period starts with the sample at 77 K, the sample histories are not the same at that point. In Fig. 2B (■) the effect can be seen of the slower cooling rates at higher T_f; slower cooling results in larger ice crystallites, and hence greater local fluoride concentration with increased extent of complex formation (as treated in [10]). Apart from the latter effect, the smooth dependence of the ratios upon the equilibration (freezing and annealing bath) temperatures is similar for the two sets of data shown in Fig. 2B. Freezing at intermediate temperatures does not noticeably affect the warming-up behavior, and annealing at increasing temperatures does not reveal a sharp transition to the limiting extent of complex formation. Thus, for the conditions of the experiments reported here, freezing and annealing with baths in the temperature range between 77 and 250 K appear not to produce transitions in the solvent phase of the frozen solution which would have a significant effect upon the combination of fluoride with ferrihemoglobin, although the possibility that the temperature-jumps are accompanied by some component of irreversible transition cannot be ruled out.

The experiments shown in Fig. 3 were performed further to investigate possible transitions in the solvent phase of frozen solutions of differing solute composition, and to ascertain what effects these transitions might have upon the exchange of fluoride for the iron-bound water molecule in ferrihemoglobin during annealing. During 'recrystallization' of a metastable aqueous frozen system, small crystals grow to be larger and thermodynamically stable ones. As a result, the semitransparent frozen solution suddenly becomes opaque as warming brings it into the rather narrow range of temperature over which recrystallization occurs. Opaqueness resulting from recrystallization was observed with the optical apparatus shown in Fig. 1 of [10]; this apparatus is more sensitive to changes in light scattering than to color change. The aqueous solution in a polyethylene EPR sample cell was frozen in liquid nitrogen and then moved to the VTC wherein the temperature of the bath of nitrogen gas was raised from 120 to 280 K in 30 minutes. Optical transmission through the frozen cell, Fig. 3A, is expected to decrease at the recrystallization temperature, T_r, and increase at the melting temperature, T_m. In the control experiment, record (a) of Fig. 3A, the onset of recrystallization of frozen phosphate buffer (in the absence of ferrihemoglobin and KF) took place at $T_r = 268$ K, and melting occurred in a narrow range of temperatures close to 273 K (as expected). With ferrihemoglobin and KF in the buffer solution, record (b) of Fig. 3A, the optical transmission decreased in two distinct temperature ranges. At and above 212 K, the frozen solution changed color from orange to dark green because of the additional combination of fluoride

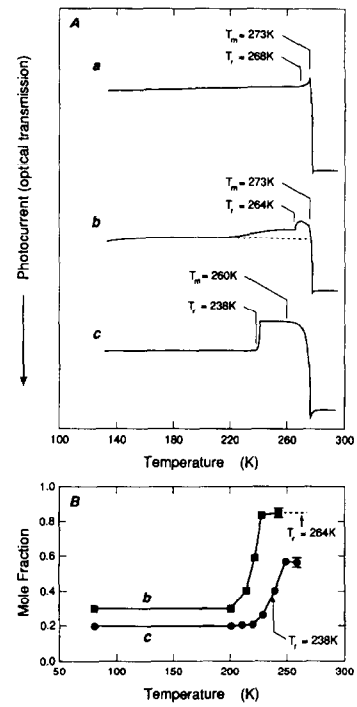

Fig. 3. (A) Recrystallization in the frozen solutions monitored by optical transmission. The sample was first frozen in liquid nitrogen, and the cell was then transferred to the VTC wherein the temperature of the flowing nitrogen gas was raised from 120 to 280 K over a period of 30 minutes. The plots show the accompanying change in optical transmission of (a) 0.033 M, pH 6.3, phosphate buffer solution; (b) the same solution as in (a) but with ferrihemoglobin ([heme] = 0.247 mM) and 1.18 mM KF; (c) the same solution as in (b) but with 48% (1.4 M) sucrose. T_r is the recrystallization temperature and T_m, the melting temperature. (B) Mole fractions from EPR measurements on frozen solutions (b)(■) and (c)(●). The procedures and instrument settings are the same as for Fig. 1. The T_r for these solutions, indicated by arrows, are from part (A) of this figure.

with ferrihemoglobin. Because a decrease in optical transmission at 212 K did not occur in the absence of ferrihemoglobin and KF, as shown in the control experiment (and indicated by the dashed line segment in (b)), no transition of the solvent phase is assigned to 212 K. Rather, the decrease in photocurrent is associated with the color change. At 264 K, opaqueness of the frozen solution increased sharply as crystallization started. Melting again occurred close to 273 K. With 48% (1.4 M) sucrose in the buffer solution in addition to ferrihemoglobin and KF, record (c) Fig. 3A, recrystallization started at 238 K, and gradual melting at about 260 K. Results of EPR measurements made on the latter two solutions are shown in Fig. 3B. The onset of the additional binding of fluoride to ferrihemoglobin at around 200 K, seen in both records (b) (no sucrose) and (c) (48% sucrose) of Fig. 3B, does not have an obvious connection with the recrystallizations monitored by optical transmission when no sucrose is present (T_r = 264 K) or when 48% sucrose is added to the reaction mixture (T_r = 238 K), (nor in frozen buffer alone, T_r = 268 K).

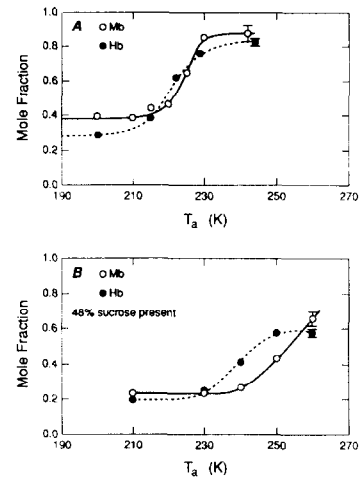

Fig. 4. Rate-limited extent (mole fraction) of fluoride complex formation, as a function of annealing temperature, in ferrimyoglobin compared with ferrihemoglobin. The procedure is the same as in Fig. 1A, the EPR settings are as in Fig. 1B, and 33 mM PO_4, pH 6.3 buffer was used. (A) Reactions of ferrimyoglobin, [heme] = 0.170 mM, (O) and ferrihemoglobin, [heme] = 0.245 mM, (●) with fluoride, [KF] = 1.23 mM. (B) Reactions of ferrimyoglobin, [heme] = 0.253 mM, (O) and ferrihemoglobin, [heme] = 0.247 mM, (●) with fluoride, [KF] = 1.18 mM in the presence of 48% sucrose.

3.3. Effects of solvent viscosity and protein structure upon the time scale of the reaction at low temperature

The viscosity of pure water and dilute aqueous solutions is about 0.01 P (P:poise = g s^{-1} cm^{-1}) at room temperature. The viscosity of unfrozen water has been extrapolated from room temperature to the glass transition temperature of around 120 K by Pryde and Jones [34], and is estimated by them to be 1 to 10 P in the temperature range 200–230 K. We now make two assumptions: (1) the 'unfrozen' water in the solvation layer of a protein has for a lower limit to its effective viscosity that of bulk water as estimated by Pryde and Jones, (a lower limit because the water molecules are restricted to move in the layer, and there they interact with polar and charged surface residues); (2) the approximate relation (viscosity) · (diffusivity) = (constant) · (absolute temperature) (Stokes–Einstein relation) holds down to the glass transition temperature. It follows that the bimolecular, diffusion-controlled, limiting rate constant in aqueous solution at room temperature, 10^{10} s^{-1} M^{-1} [35], drops to 10^8 or 10^7 s^{-1} M^{-1} (possibly lower in the hydration layer of protein) in the temperature range 200–230 K, but is orders of magnitude greater than is suggested by Fig. 1C and Fig. 2B. From more direct kinetic experiments, see next section and Fig. 5, the bimolecular rate constant for the additional binding of fluoride to ferrihemoglobin is measured to be about 10^{-2} s^{-1} M^{-1} at temperatures between 200 and 230 K. Clearly the rate of exchange of fluoride for water at nearby heme sites is not diffusion-controlled.

Fig. 4 shows that ferrimyoglobin and ferrihemoglobin replace water with fluoride at different rates at the same temperature, indicating that, whatever the role of the solvent, the rates are protein specific. Fig. 4A shows the extent of complex formation in the two proteins in the absence of sucrose, and Fig. 4B demonstrates the effect of 48% sucrose upon complex formation. Fig. 4 clearly demonstrates that the association rate is not diffusion-controlled because a diffusion-controlled reaction would be dependent upon the diffusion rate of the ligand and not

upon the protein itself. The difference between ferrimyoglobin and ferrihemoglobin seen in Fig. 4 indicates further that these proteins are not denatured by the cold [36] because, if they were denatured, the reaction rates would be expected not to differ in their dependence upon temperature.

3.4. Kinetic studies of the replacement of heme-bound water by fluoride in ferrimyoglobin and ferrihemoglobin at subfreezing temperatures

The experimental results reported above show that there is a useful range of temperature in which

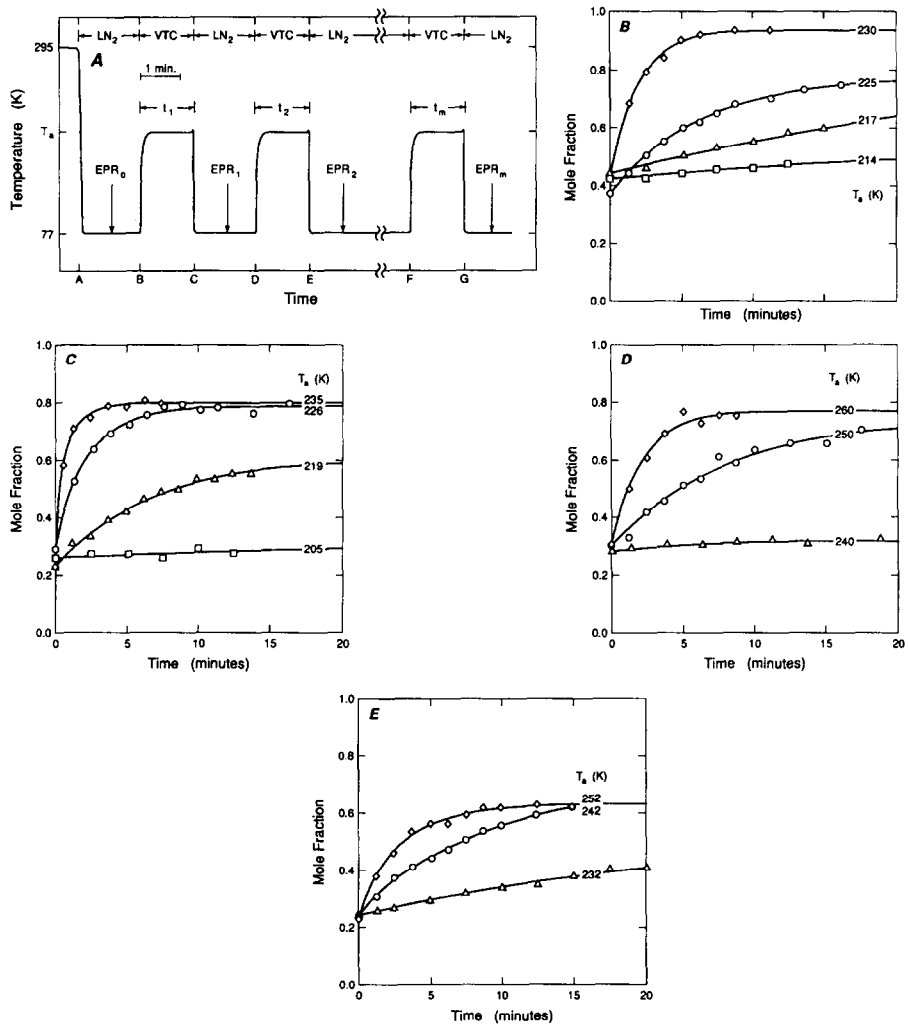

Fig. 5. Kinetic curves for the combination of ferrimyoglobin and ferrihemoglobin with fluoride after jumping the temperature from 77 K to various annealing temperatures. (A) The procedure for the temperature jump experiments is shown. At time A the sample cell was immersed in liquid nitrogen. The temperature of the sample was then raised in a bath of gas at annealing temperature T_a for a period of time t_1 starting at B, for an additional period of time t_2 starting at D, etc. EPR spectra were measured at 77 K after each annealing period (i.e. C, E, etc.). The EPR instrument settings are the same as in Fig. 1. Kinetic curves are shown for (B) the combination of ferrimyoglobin (0.170 mM) with fluoride ([KF] = 1.23 mM) in 0.033 M PO_4, pH 6.3 buffer at temperatures (T_a) 230, 225, 217 and 214 K; (C) the combination of ferrihemoglobin ([heme] = 0.245 mM) with fluoride ([KF] = 1.23 mM) in the same buffer at temperatures 235, 226, 219 and 205 K; (D) the combination of ferrimyoglobin (0.253 mM) with fluoride ([KF] = 1.18 mM) in the same buffer plus 48% sucrose at temperatures 260, 250 and 240 K; (E) the combination of ferrihemoglobin ([heme] = 0.247 mM) with fluoride ([KF] = 1.18 mM) in the same buffer as in (D) (with 48% sucrose) at temperatures 252, 242 and 232 K. The lines are least squares fits to Eq. 3.

transitions in the solvent phase of the frozen solution do not have a significant effect upon the local concentration of fluoride or upon the exchange of this ligand for water at nearby heme sites, and show further that the rates of this process in ferrimyoglobin and ferrihemoglobin are not diffusion-controlled. With their feasibility in this temperature range established, kinetic studies of the reactions in ferrimyoglobin and ferrihemoglobin were carried out, Fig. 5. The experimental protocol is depicted in Fig. 5A where t_n, $n = 1$ to m, is the nth period of time when the polyethylene EPR sample cell was placed in the dewar of the VTC. Because 0.3 min is required for the temperature of the sample to reach 95% of the jump from 77 K to T_a, the time for each annealing period is taken as $t_n - 0.3$ min. The mole fraction of complex $x_n(t)$ present after the nth annealing period is calculated with the EPR signal amplitude measured just after that period; x_n is a function of the total time that the sample has been in the VTC at temperature T_a, which is $t = \Sigma_{i=1,n}(t_i - 0.3)$ (min). The time courses for the combination of fluoride with ferrimyoglobin at various temperatures T_a are shown in Fig. 5B. Analogous kinetic curves for the combination of fluoride with ferrihemoglobin are presented in Fig. 5C. The conditions of the experiments for the kinetic curves in Fig. 5D and E are the same as those for Fig. 5B and C except that 48% sucrose was added to the hemeprotein and KF in buffer; in view of the limited number of kinetic measurements with sucrose present, these results should be viewed as qualitative.

In the frozen solutions used in these kinetic studies, the average number of fluoride ions nearby and available for binding to a heme site is large, but only one is required to replace the water molecule of what was the aquo complex. The local concentrations of fluoride and water are large and not significantly affected by the loss or gain of a single ion or molecule. Therefore the overall mass action relation associated with the fluoride for water exchange reaction (a more complex process than ligand binding to the pentacoordinate ferrous sites in deoxy-myoglobin and hemoglobin) can be modeled as pseudo-first order in the heme complexes. In Eq. 1, which is a phenomenological description of the overall rate process leading to equilibrium and does not include all steps of the kinetic pathway, k_1 and k_{-1} are second order rate constants and x is the mole fraction of fluoride complex.

$$F^- + \underset{1-x}{\text{heme} \cdot H_2O} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H_2O + \underset{x}{\text{heme} \cdot F^-}. \qquad (1)$$

This description has previously been used to interpret a large amount of kinetic data from reactions of ligands with ferrihemoglobin and ferrimyoglobin [37]. Although kinetic studies of carbonyl complexes of ferrous hemeproteins implicate intermediates in the process of CO dissociation, no similar studies of the fluoride complexes of ferrimyoglobin and ferrihemoglobin are reported in the literature. Therefore, for interpretation of the measurements at cryogenic temperatures presented here, the well-established assumptions implicit in Eq. 1 are followed.

If the second order rate constant k_{-1} is multiplied by the water concentration to give the pseudo-first order rate constant k'_{-1}, then the (net) rate of formation of the fluoride complex is

$$dx/dt = k_1 \cdot [F^-] \cdot (1 - x) - k'_{-1} \cdot x. \qquad (2)$$

When k_1 and k'_{-1} are obtained from kinetic measurements, the equilibrium constant $K = k'_{-1}/k_1$ can be calculated. The solution of differential Eq. 2 is

$$x(t) = x_L + [x_0 - x_L] \cdot \exp[-(k_1 \cdot [F^-] + k'_{-1})t] \qquad (3)$$

with

$$x_L = k_1 \cdot [F^-]/(k_1 \cdot [F^-] + k'_{-1});$$

x_0 is the mole fraction of fluoride complex present before the temperature jump, and x_L is the limiting value of the mole fraction following the post-temperature jump transient.

Eq. 3 was fit to the kinetic data of Fig. 5B–E with a computer program based upon the non-linear least squares algorithm of Marquardt to obtain the rate constants k_1 and k'_{-1}. Note that, in Eq. 3, k_1 and $[F^-]$ appear only as their product, and the values of this product are what is obtained from fitting the kinetic curves. To arrive at k_1 from a $k_1 \cdot [F^-]$ product, independent knowledge of the local fluoride concentration is required; this concentration is estimated by the method of [10] to be 70 mM in the absence of sucrose, and 20 mM when 48% sucrose is present. The results for ferrimyoglobin are given in

Table 1
Rate constants for the formation and dissociation of the fluoride complex of ferrimyoglobin

	Temperature (K)	k_1 ($s^{-1} M^{-1}$)	k'_{-1} (s^{-1})
[sucrose] = 0.0; [heme] = 0.170 mM; $[F^-]_{ave}$ = 1.23 mM; $[F^-]_{local}$ = 70 mM	230	$1.1 \cdot 10^{-1}$	$5.2 \cdot 10^{-4}$
	227	$6.4 \cdot 10^{-2}$	$7.3 \cdot 10^{-4}$
	225	$2.8 \cdot 10^{-2}$	$5.8 \cdot 10^{-4}$
	223	$1.9 \cdot 10^{-2}$	$3.0 \cdot 10^{-4}$
	217	$5.6 \cdot 10^{-3}$	$5.9 \cdot 10^{-6}$
	214	$1.6 \cdot 10^{-3}$	$2.0 \cdot 10^{-6}$
[sucrose] = 1.4 M; [heme] = 0.253 mM; $[F^-]_{ave}$ = 1.18 mM; $[F^-]_{local}$ = 20 mM	260	$2.8 \cdot 10^{-1}$	$1.7 \cdot 10^{-3}$
	250	$8.0 \cdot 10^{-2}$	$4.8 \cdot 10^{-4}$
	240	$2.6 \cdot 10^{-2}$	$9.1 \cdot 10^{-4}$

Table 1. Kinetic curves for ferrihemoglobin showed structured residuals when fit by a single reaction site (i.e. one forward rate constant, and one reverse); simulations with two sites (x_L taken the same) matched the experimentally observed kinetic curves well. The simplest explanation for the more complex kinetic behavior of hemoglobin is that differences between the α- and β-subunits give rise to differences in rate constants; however, in the time course of the reaction of fluoride with the isolated chains at 20°C, no difference was found between α and β [37]. We shall discuss the results for ferrihemoglobin, given in Table 2, in terms of sites a and b (which may or may not correspond to α- and β-subunits). Note in Table 2 that the differences between the corresponding a and b rate constants are not great.

4. Discussion

Fig. 6 shows $\Delta G°$ in ice calculated from the entries of Tables 1 and 2 by means of $K = k'_{-1}/k_1$ and also $\Delta G°$ extrapolated (dashed lines) to subfreezing temperatures from $\Delta G°(T)$ (solid lines) determined by measurements upon solutions above freezing. One sees in Fig. 6 that the $\Delta G°$ measured below freezing temperature are generally smaller than the extrapolated values. The measured $\Delta G°$ values also decrease with decreasing temperature; the tempera-

Table 2
Rate constants for the formation and dissociation of the fluoride complex of ferrihemoglobin

	Temperature (K)	Site a		Site b	
		k_1 ($s^{-1} M^{-1}$)	k'_{-1} (s^{-1})	k_1 ($s^{-1} M^{-1}$)	k'_{-1} (s^{-1})
[sucrose] = 0.0; [heme] = 0.245 mM; $[F^-]_{ave}$ = 1.23 mM; $[F^-]_{local}$ = 70 mM	235	$1.7 \cdot 10^{-1}$	$3.1 \cdot 10^{-3}$	$5.4 \cdot 10^{-1}$	$9.6 \cdot 10^{-3}$
	228	$7.3 \cdot 10^{-2}$	$1.2 \cdot 10^{-3}$	$2.0 \cdot 10^{-1}$	$3.2 \cdot 10^{-3}$
	226	$5.8 \cdot 10^{-2}$	$1.1 \cdot 10^{-3}$	$1.4 \cdot 10^{-1}$	$2.7 \cdot 10^{-3}$
	224	$3.0 \cdot 10^{-2}$	$6.9 \cdot 10^{-4}$	$7.9 \cdot 10^{-2}$	$1.8 \cdot 10^{-3}$
	222	$3.2 \cdot 10^{-2}$	$9.6 \cdot 10^{-4}$	$4.9 \cdot 10^{-2}$	$1.4 \cdot 10^{-3}$
	220	$9.6 \cdot 10^{-3}$	$1.6 \cdot 10^{-4}$	$5.8 \cdot 10^{-2}$	$9.4 \cdot 10^{-4}$
	219	$2.1 \cdot 10^{-2}$	$9.6 \cdot 10^{-4}$	$2.1 \cdot 10^{-2}$	$9.6 \cdot 10^{-4}$
	214	$1.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-3}$	$1.3 \cdot 10^{-2}$	$1.1 \cdot 10^{-3}$
	205	$7.7 \cdot 10^{-4}$	$1.6 \cdot 10^{-5}$	$7.7 \cdot 10^{-4}$	$1.6 \cdot 10^{-5}$
[sucrose] = 1.4 M;[heme] = 0.247 mM; $[F^-]_{ave}$ = 1.18 mM; $[F^-]_{local}$ = 20 mM	252	$1.5 \cdot 10^{-1}$	$1.7 \cdot 10^{-3}$	$2.4 \cdot 10^{-1}$	$2.7 \cdot 10^{-3}$
	242	$2.4 \cdot 10^{-2}$	$1.1 \cdot 10^{-4}$	$1.1 \cdot 10^{-1}$	$5.1 \cdot 10^{-4}$
	232	$1.4 \cdot 10^{-2}$	$1.5 \cdot 10^{-4}$	$1.4 \cdot 10^{-2}$	$1.5 \cdot 10^{-4}$

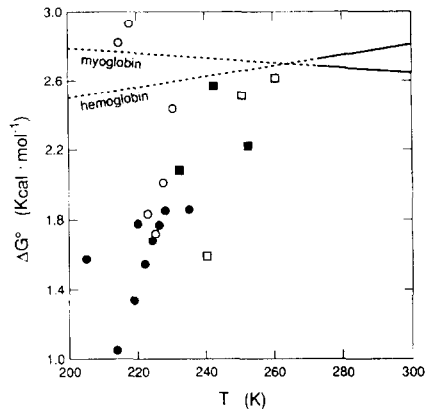

Fig. 6. Temperature dependence of the standard free energy change accompanying the dissociation of the fluoride complexes of ferrimyoglobin and ferrihemoglobin. The entries between 205 and 260 K are calculated with $\Delta G^\circ = -RT \ln(k'_{-1}/k_1)$ from the rate constants listed in Tables 1 and 2: (O) ferrimyoglobin, no sucrose; (●) ferrihemoglobin, no sucrose; (□) ferrimyoglobin, 48% sucrose present; (■) ferrihemoglobin, 48% sucrose present. The lines which are solid above and dashed below 273.2 K are calculated from ΔH° and ΔS° measured above freezing.

ture dependence shown in Fig. 6 indicates that, if the change in enthalpy were independent of temperature, the entropy change would be much more negative in the subfreezing region than above freezing. However, because information about the enthalpy change for ligand dissociation below freezing temperature is not available, the following discussion is limited to the free energy change, ΔG°. With regard to ΔG° for horse ferrimyoglobin–fluoride in 33 mM PO_4, pH 6.3 buffer, the enthalpy and entropy of dissociation measured above freezing temperature in this laboratory are 3.07 kcal/mol (12.8 kJ/mol) and 1.3 cal/(mol K) (5.3 J/(mol K)), respectively. As can be seen in Fig. 5B–E, the time course shows little curvature at the lowest temperatures of each series; it follows that the rate constants, the asymptotic values of mole fraction, and the dissociation constant (K) are not as well-determined at the lowest temperatures as at temperatures above these. The dissociation constant K_{extrap} at each subfreezing temperature is readily calculated with ΔH° and ΔS° measured above freezing. One can then compare the extrapolated with the experimentally-obtained binding at each temperature by means of the ratio K_{extrap}/K. Including the data from both myoglobin and hemoglobin, without and with sucrose present, and giving equal weight to every value of K independent of temperature, one finds the average $\langle K_{extrap}/K \rangle = 0.37$. On this basis, fluoride binding at subfreezing temperatures is about 3 times weaker than calculated with thermodynamic parameters obtained above freezing; i.e. freezing has only a small effect (about -0.7 kcal/mol in ΔG°) upon the temperature dependence of the stability of the fluoride complexes of ferrimyoglobin and ferrihemoglobin observed above freezing.

Departing from the rigorous treatment of the data given above, for exploratory purposes one can analyze the temperature dependence of the phenomenological rate constants according to the Arrhenius-related formulas

$$\ln k_1 = f_1 - \Delta H_1^*/RT \quad (4)$$

and

$$\ln k'_{-1} = f'_1 - \Delta H^*_{-1}/RT \quad (5)$$

where k_1 (second order rate constant in the direction of formation of the fluoride complex) and k'_{-1} (first order rate constant in the reverse direction) are expressed in terms of process frequency factors f_1 and f'_{-1} (which contain an activation entropy factor), and process activation energies ΔH_1^* and ΔH^*_{-1}. There

Table 3
Quantification of temperature dependence of rate constants; parameters obtained from linear least squares fits to $\ln k_i = f_i - \Delta H_i/RT$

Parameter	From $\ln k_1$ vs. $1/T$			From $\ln k'_{-1}$ vs. $1/T$		
	myoglobin	hemoglobin a	hemoglobin b	myoglobin	hemoglobin a	hemoglobin b
f_i	53	34	44	82	25	35
f_i, sucrose	27	25	32	0.3	21	28
ΔH_i^*	25	17	21	40	14	18
ΔH_i^*, sucrose	15	14	17	4	14	17

The enthalpy units are kcal/mol.

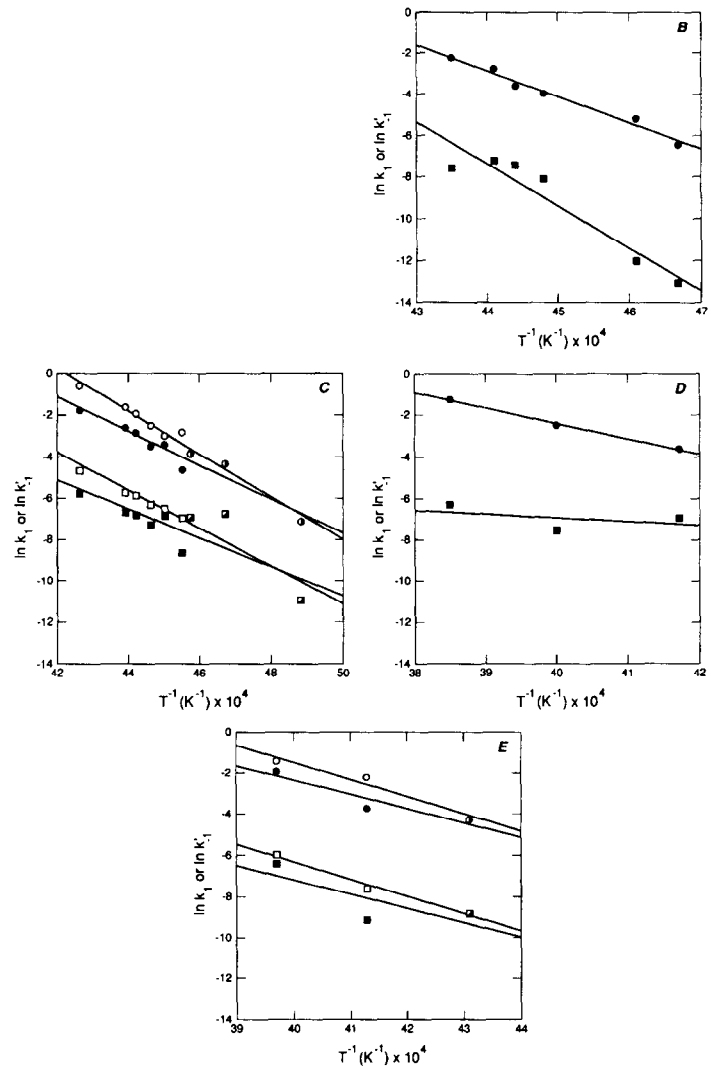

Fig. 7. Arrhenius plots of temperature dependence of forward and reverse rate constants obtained from the kinetic curves of Figs. 5B–E. The straight lines are linear least squares fits to the data of Tables 1 and 2. Fig. 7B–E correspond to Fig. 5B–E; there is no Fig. 7A. (B) Ferrimyoglobin: (●) forward; (■) reverse. (C) Ferrihemoglobin: (●) forward, a; (O) forward, b; (■) reverse, a; (□) reverse, b. (D) Ferrimyoglobin in 48% sucrose: (●) forward; (■) reverse. (E) Ferrihemoglobin in 48% sucrose: (●) forward, a; (O) forward, b; (■) reverse, a; (□) reverse, b.

can be some temperature dependence in the frequency factors [17] and this would be included in the $\Delta H^*/RT$ terms. For the short temperature ranges of the experiments, (16 K for ferrimyoglobin without sucrose, 30 K for ferrihemoglobin without sucrose, and 20 K for both proteins with sucrose present), formulas 4 and 5 provide satisfactory fits to the data, as shown in Fig. 7. The results are given in Table 3. The numbers in Table 3 from the sucrose-containing solutions are not quantitatively accurate because the amount of sucrose data is limited and because there is uncertainty in activation energies estimated from measurements over such a narrow range of temperatures. However, qualitatively they reveal interesting features of the exchange process. For each of the ferrihemoglobin sites, Table 3 shows that the activa-

tion enthalpies for the forward and reverse processes differ little when sucrose is absent, and less when sucrose is present. (The differences between the forward and reverse activation enthalpies, and differences between the frequency factors, are the same for the a and b sites, without and with sucrose present; this follows from taking x_L to be the same for a and b). The frequency factors have greater influence than the activation energies upon the formation of the ferrihemoglobin fluoride complex. The differences between the activation enthalpies of the forward and reverse processes are greater for ferrimyoglobin than for ferrihemoglobin.

As noted above, the temperature dependence of the phenomenological rate constants is more complex than given by Eqs. 4 and 5. With this limitation in mind, it is instructive to calculate, by means of the parameters of Table 3, the values of k_1 in ice to projected values above freezing; the latter can then be compared with k_1 values obtained by measurements (in other laboratories) above freezing. The experimentally-obtained k_1 value for horse ferrimyoglobin in solution at 295 K, pH 6.05, is 20 s^{-1} M^{-1} [38]; the values projected to 295 K are $2 * 10^4$ s^{-1} M^{-1} on the basis of measurements over the range 214–230 K with sucrose absent, and 8 s^{-1} M^{-1} for the range 240–260 K with 48% sucrose present. The same behavior is shown by the hemoglobin subunits. The experimentally-obtained k_1 value for human ferrihemoglobin (subunit average) at 293 K, pH 6.09, is 26 s^{-1} M^{-1} [37]; for subunit a at 295 K, k_1 is projected to be 290 s^{-1} M^{-1} based upon measurements over the range 205–235 K with sucrose absent and 6 s^{-1} M^{-1} for the range 232–252 K with 48% sucrose present; for subunit b, $6.8 \cdot 10^3$ s^{-1} M^{-1} with sucrose absent and 39 s^{-1} M^{-1} with 48% sucrose present. Note that k_1 values projected from the measurements on sucrose-containing frozen solutions are close to those measured at the physiological temperature, whereas k_1, values projected from sucrose-free frozen solutions are much greater. Sucrose is known to reduce the size of the ice crystallites in frozen aqueous solution, and 48% sucrose appears to enable the temperature-dependence of the rate constant measured in ice to express, at least qualitatively, the value above freezing. These two observations, together with the large difference in k_1 when sucrose is absent from the ice, suggest that the large ice crystallites in frozen aqueous solution not containing cryoprotectant are responsible for a significant effect upon the rate of exchange of fluoride for water at the iron. Apparently, provided freezing conditions are such that ice crystallites have not grown large, the rates of fluoride for water replacement in ferrimyoglobin and ferrihemoglobin are largely unaffected by the passage from aqueous liquid to ice.

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