EFFECTS OF AMINO ACIDS ON GELATION KINETICS AND SOLUBILITY OF SICKLE HEMOGLOBIN

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SUMMARY: Ten amino acids have been studied for their effects on the gelation of sickle hemoglobin using the recently developed assay of Hofrichter, Ross and Eaton. By monitoring kinetics and using high speed sedimentation, the rate and extent of gelation are directly measured. Of the amino acids tested, only phenylalanine significantly inhibited the gelation of sickle hemoglobin. The systematic study of the effects of additives, such as amino acids, on gelation serves as a basis for the study of potential non-covalent inhibitors of sickling.

In the last few years, measurements which give additional information about molecular mechanisms of sickling such as solubility in high salt concentrations (1), ultracentrifugation equilibrium (2), and oxygen affinity of hemoglobin S (3) have supplemented the cell sickling and minimum gelling concentration (MGC) (4) methods for studying potential antisickling agents. Using these methods, a number of chemical agents which interact non-covalently with sickle hemoglobin, such as guanidine hydrochloride (5), urea (5,6), alkylureas (7), extract of Fagara xanthoxyloides (8) and 3,4-dihydro-2,2-dimethyl-2H-1 benzopyran-6-butyric acid (9) have been studied. Additionally, the amino acids L-asparagine, L-glutamine, L- and DL-homoserine, L-lysine and L-arginine have been found to inhibit erythrocyte sickling (10-12). The first three of these amino acids have also been reported to increase the solubility of hemoglobin S in 1.4 M phosphate (13). Recently, peptide fragments from hemoglobin S (β^S(1-6), β^S(1-3), β^S(5-6)) and hexa-L-prolineamide have been reported to increase the MGC (14).

A simple technique for directly assaying the equilibrium composition of the gelled sample was described by Bertles (15) in which the polymerized hemoglobin is pelleted by high speed sedimentation and the concentration of hemoglobin in the supernatant is assayed spectrophotometrically. Hofrichter,

Ross, and Eaton have studied the kinetics of gelation by a variety of physical techniques (16,17), as have Malfa and Steinhardt (18) and Moffat and Gibson (19). Recently Hofrichter et al. (20,21) have introduced the use of small, cylindrical, sealed quartz tubes for measurements of both the kinetics of gelation (by turbidometry) and of hemoglobin solubility on the same sample. Thus the rate and extent of gelation - the two solution properties which presumably will correlate best with the sickling processes - can be measured directly. We have begun a systematic study of amino acids and peptides as potential stereospecific inhibitors of gelation using the Hofrichter, Ross and Eaton assay.

MATERIALS AND METHODS

Hemoglobin S was purified from lysates of erythrocytes of sickle disease patients (22), concentrated by ultrafiltration and dialyzed against 0.15 M potassium phosphate buffer, pH 7.35. L-amino acids were dissolved in the buffer and final pH adjusted to 7.35 with HCl or KOH. The hemoglobin samples were deoxygenated under nitrogen with sodium dithionite (0.05 M) and sealed in quartz EPR tubes (Wilmad-PQ701).

Hemoglobin concentrations were determined in the EPR tubes spectrophotometrically using optical densities at 910 nm and 1090 nm ([HB-S] = (O.D. 910 - O.D. 1090 + 0.04) 24.56 gm/100 ml) as described by Hofrichter et al. (20). The delay time t_d, as determined by turbidity measurements at 800 nm, is the time between the temperature jump (from ice bath to 20°C or 30°C) and the beginning of turbidity increase. The solubility or supernatant concentration is measured after ultracentrifugation at 100,000 x G for 3 hours of the gelled samples.

RESULTS

The total hemoglobin concentration in the samples varied from 22.9 to 27.7 gm/100 ml. The solubility of deoxyhemoglobin S in the control samples (no amino acids) was 16.54 gm/100 ml at 30°C with a standard deviation of 0.22 gm/100 ml (1.3%). The values for the solubility of hemoglobin S in the presence of the ten amino acids (Fig. 1) fall within two standard deviations of the solubility for the control samples with the exception of phenylalanine. Phenylalanine samples also had corresponding longer delay times. For example, for an initial hemoglobin concentration of 23 gm/100 ml at 30°C, as the phenylalanine concentration increases from 0 to 32 mM

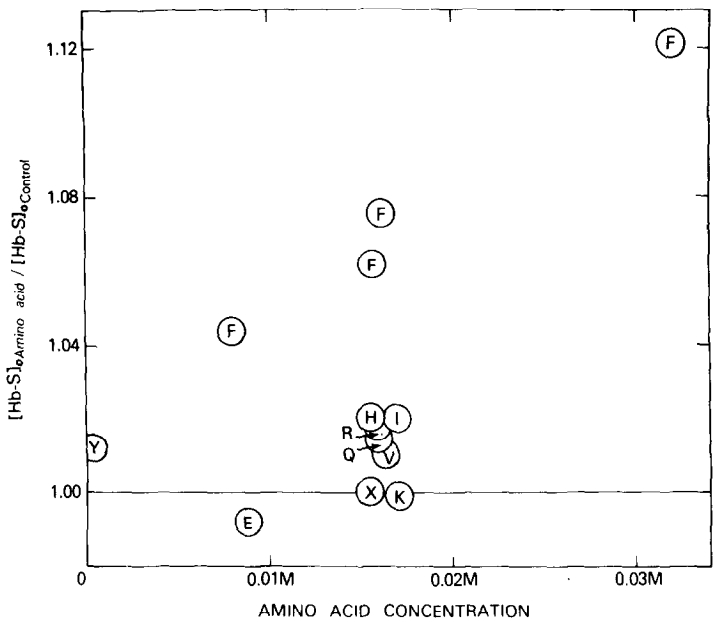

Figure 1. Effect of amino acid type and concentration on solubility at 30°C. The data is expressed as a ratio to the control solubility determined simultaneously for each set of data (each data point represents duplicate samples except for phenylalanine at 16 mM, which represents a single sample). The one-letter amino acid abbreviations are taken from Ref. 29. E, Glu (Fox Chem. Co.); F, Phe (Fox Chem. Co.); H, His (Sigma); I, Ile (Fox Chem. Co.); K, Lys (Fox Chem. Co.); Q, Gln (Fox Chem. Co.); R, Arg (Mann Resrch. Lab.); V, Val (Fox Chem. Co.); X, homoserine (CalBiochem); Y, Tyr (CalBiochem). All amino acids used were the L isomer.

the solubility increases from 16.5 to 18.6 gm/100 ml (solubility normalized to average control solubility), and the delay time increases by 14-fold.

Plots of the logarithm of the delay time t_d versus the supersaturation ratio ($S \equiv [HB\text{-}S]/[HB\text{-}S]_o \equiv$ (total concentration/solubility)) at 20°C (Fig. 2) and 30°C (data not shown) can both be fitted with a straight line. This result confirms the suggestion (16) that the delay time varies directly with the supersaturation ratio to the nth power ($1/t_d \propto S^n$). This relationship has been shown to hold for the inhibition of gelation by temperature, urea, CO, and pH changes (20) and our results indicate that this is additionally true for amino acids which inhibit gelation.

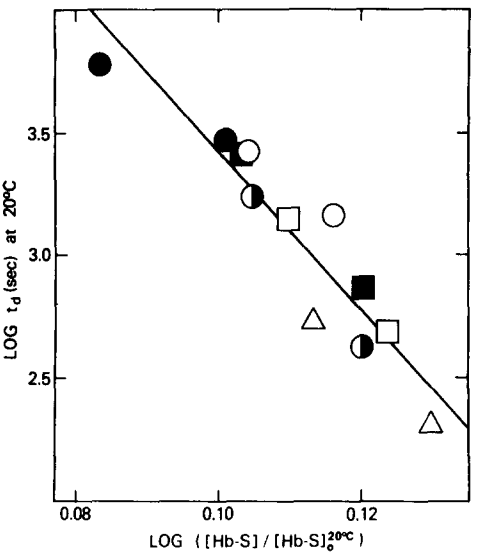

Figure 2. The supersaturation ratio (S ≡[HB-S]/[HB-S]o) as a function of the delay time t_d at 20°C. The straight line (log t_d = -31.9 log S + 6.6) was determined by a linear regression analysis (r^2 = .867) of the data (controls and amino acid samples with varying concentrations with $[HB-S]_o$ taken to be the average solubility of duplicate samples.) These results indicate that the relationship $1/t_d = \gamma S^n$ is independent of amino acid type or concentration. Δ, control; ○, phenylalanine with [Phe] = 8 mM; ◑, [Phe] = 16 mM; ●, [Phe] = 32 mM; □, tyrosine with [Tyr] = 0.26 mM; ■, [Tyr] = 0.52 mM.

DISCUSSION

We believe that our results confirm the value of the assay and theory developed by Hofrichter et al. (16,17,20,21) as a description of the thermodyanamics and kinetics of gelation and as a valuable method for testing potential inhibitors under conditions as close to "physiological" as now possible. Of the amino acids we have tested so far, phenylalanine has the largest effect as an inhibitor, which, in fact, is about 2.5 times that of urea on a molar basis. If the phenylalanine effect continues to be linear at higher concentrations, then a 100-fold increase in delay time, a change which might be sufficient to produce improvement in clinical status (21), would be expected at a concentration of 60 mM (see below). Tyrosine may have a similar effect on a molar basis to phenylalanine but its low solubility

limits further study. The other amino acids, including several reported as inhibitors by others, have much smaller, if any, effects.

The effect of phenylalanine on gelation may be non-specific due to solvent interactions characteristic of aromatic rings or may be stereospecific. It is interesting that the β^{6}-valine is in close intermolecular contact with β^{85}-phenylalanine in the deoxyoxyhemoglobin S crystal (23), raising the possibility that the phenylalanine inhibition effect is stereospecific.

We have been investigating these amino acid additives as a preliminary to the study of other macromolecular perturbants, especially oligopeptides. This work is primarily based on the hypothesis that peptides from the sequence of the α and β^{S} chains of sickle hemoglobin, in the inter-tetramer contact regions, may have sufficient occupancy of native-like conformations to behave as specific inhibitors of polymerization (24).

Non-covalent inhibitors of gelation have possible advantages over covalent-modification reagents, such as potassium cyanate (25), dimethyl adipimidate (26), and nitrogen mustards (27), with respect to toxicity, changes in oxygen affinity and other factors. However, the therapeutic use of any such compounds may be limited by low erythrocyte membrane permeability and/or required high dosages. Phenylalanine, for example, has shown severe toxicity in the millimolar concentration range (28). In any case, however, these studies should help to clarify the nature and energies of intermolecular contacts in the polymerization of sickle hemoglobin.

We are now pursuing other studies of the interaction of L-phenylalanine and related compounds with sickle hemoglobin, as well as gelation studies with peptides.

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