Biochemical Pharmacology, Vol. 35, No. 2, pp. 317–323, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

EFFECT OF GLUTARALDEHYDE ON HAEMOGLOBIN: OXIDATION–REDUCTION POTENTIALS AND STABILITY

DIDIER GUILLOCHON, LAURENT ESCLADE and DANIEL THOMAS
Laboratoire de Technologie Enzymatique, E.R.A. No 338 du CNRS,
Université de Technologie de Compiègne, 60206 Compiègne, France

(*Received* 12 *April* 1985; *accepted* 1 *August* 1985)

Abstract—Glutaraldehyde is a reagent widely used for the cross-linking of haemoglobin for use as a blood substitute. Most of the previous studies were limited to oxygen binding equilibria of the glutaraldehyde-modified haemoglobin. This paper concerns the impact of glutaraldehyde on oxidation–reduction equilibria, autoxidation kinetics and stability towards heat and urea of haemoglobin cross-linked in the oxy, deoxy and ferri states. The oxidation–reduction potentials and homotropic effects were reduced; however, the oxidation Bohr effect was not significantly different when compared with native haemoglobin. Haemoglobin immobilized in the oxy or ferri state exhibited a lower redox potential than when immobilized in the deoxy state. The autoxidation rates were increased after cross-linking, particularly at basic pH. Cross-linking stabilizes ferrihaemoglobin better than oxy or deoxyhaemoglobin against thermal- and urea-induced denaturation. Glutaraldehyde cross-linking does not stabilize haemoglobin against urea-denaturation. The experimental results were interpreted as indicating a chemical modification of the protein without 'conformation freezing' and by an opening of the haem pocket to the aqueous solvent.

Glutaraldehyde is a widely-used reagent for the cross-linking of haemoglobin in the context of blood-substitutes research [1–7]. However, in spite of its utilization in biochemistry for both fundamental and technological purposes, particularly for the immobilization of enzymes, glutaraldehyde solutions are not clearly defined and its reaction mechanism with proteins is misunderstood: glutaraldehyde solutions consist of polymers which react with a protein at many cross-linking sites and give rise to a heterogeneous population of cross-linked monomers and polymers [8–15].

Many previous studies of glutaraldehyde action on haemoglobin have focused mainly on oxygen binding. However, the appraisment of the utilization of haemoglobin cross-linked by glutaraldehyde as blood substitute requires more knowledge of the effects of glutaraldehyde on haemoglobin. For instance it is important to appraise the redox potentials, the autoxidation rates of the iron and the stability of the glutaraldehyde-modified haemoglobin.

We have previously studied the effect of glutaraldehyde on the allosteric properties of human haemoglobin [16]. Effect of pH and 2,3-diphosphoglycerate on oxygen binding and cross-linking were studied with haemoglobin immobilized in both the oxy and deoxy states: the cooperativity is suppressed and the affinity is increased when compared with native haemoglobin; haemoglobin immobilized in the oxy state exhibited a higher oxygen affinity than that immobilized in the deoxy state. Most of the changes in behaviour of cross-linked haemoglobin are due to chemical modification without freezing of R or T conformations by cross-linking. Moreover, it was shown that haemoglobin chemically modified by glutaraldehyde (without polymerization), exhibited a behaviour similar to that of heterogeneous cross-linked soluble polymers.

The aim of this paper is to extend the knowledge of the biochemical properties of glutaraldehyde modified haemoglobin which is necessary for pharmacological or technological utilizations. This paper is a continuation of the previous study of the impact of glutaraldehyde on human haemoglobin and deals with the oxidation–reduction equilibria and the stability towards ferrous iron autoxidation and denaturation by heat and urea.

METHODS

Haemoglobin preparation. Human oxyhaemoglobin was prepared from freshly drawn blood as described previously [17]. All haemoglobin concentrations were measured according to Drabkin [18]. Ferrihaemoglobin was prepared using potassium ferricyanide in slight excess. Potassium ferricyanide and ferrocyanide were removed from the preparation using a Bio-Gel P6 column. For each set of experiments only one preparation of haemoglobin was used.

Production of cross-linked soluble polymers. A solution of 0.05 M phosphate buffer, pH 7.2 containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde was prepared. After 10 min, glycine was added to 10 mg/ml. Immobilization took place either in the presence or in the absence of oxygen. In the latter case, immobilization took place in an atmosphere of nitrogen (Air Liquide, quality U, 10 ppm oxygen), the absence of oxygen being checked by a Clark oxygen electrode for all the solution used.

Gel filtration. Filtration was performed using sepharose 6B with a bed volume of 110 ml. One-ml samples of cross-linked soluble polymers or native haemoglobin (20 mg/ml) were eluted with 0.1 M phosphate buffer pH 6.8 at a flow rate of 9 ml/hr. Molecular weights calibration of the gel was carried

out with dextran blue, thyroglobulin, pyruvate kinase (ATP: pyruvate phosphotransferase, 2.7.1.40), Aldolase (fructose-1, 6-diphosphate-D-glyceraldehyde-3 phosphate-lyase, 4.1.2.13) and native haemoglobin.

Measurements of redox potential. Oxidation–reduction equilibria were investigated by means of potentiometric titrations, performed in vessels controlled by thermostat at 25° under purified nitrogen (nitrogen, quality U, purified by bubbling in a solution of dithionite). The solution to be titrated was obtained by adding a concentrated solution of native or cross-linked haemoglobin in the ferri form to previously deoxygenated 0.1 M phosphate or borate buffer (final tetramer haemoglobin concentration 10^{-4} M). The magnetically stirred solution was flushed on the surface by purified nitrogen before and during the titration.

In order to avoid diffusional constraints on large molecules (such as anthraquinone sulfonate) inside the haemoglobin polymers, the reductant used was a solution of dithionite in the deoxygenated buffer prepared just before each titration experiment. Toluidine blue was added (3% of the haemoglobin concentration) as mediator. With this mediator, equilibria were attained within 1 min.

The reference cell was a saturated calomel electrode calibrated at 25° against a hydrogen electrode. The potential of the half-cell was 0.244 V at 25°. The Eh values were referred to the normal hydrogen electrode after the convention of Clark [19]. A CARY 401 potentiometer was used in connection with a recorder. The pO_2 was checked with a Clark oxygen electrode during each titration. The pH was checked after each redox potential measurement and spectra of haemoglobin were recorded in order to confirm the absence of hemochromes. Moreover, in order to demonstrate the absence of side effects of dithionite on the measured redox potentials, controls with native haemoglobin were performed by titrating the haemoprotein, in the ferrous and ferric states, with ferricyanide and dithionite, respectively, at different pHs. The same redox potentials were obtained.

Autoxidation rate measurements. Measurement of the autoxidation rate was carried out spectrophotometrically as described previously [20], in air-saturated 0.1 M phosphate buffer over the pH range 5.5–8 with 2 mg/ml HbO_2* in the temperature range 10–55°. The reaction was followed either by scanning over the wavelength region 650–450 nm at selected time intervals or by following the absorbance at fixed wavelengths (usually 630 nm) as a function of time. The ratio of the concentration of HbO_2 at time t = 0 to that after time t, which is required for the first order plot, was obtained by following absorbance at 630 nm and by the equation:

$$(HbO_2)_0/(HbO_2)_t = (A_0 - A_\infty)/(A_t - A_\infty),$$

where A_0, A_t and A_∞ are respectively the absorbances at time 0, t and completion of the reaction (obtained by adding a small amount of potassium ferricyanide). The pH was checked before and after each experiment and spectra of haemoglobin were recorded in order to confirm the absence of hemichromes formation during autoxidation rate measurements.

Thermal denaturation rate measurements. The kinetics of thermal denaturation of haemoglobin were measured essentially as by other authors [21]. The experiments were carried out in 0.1 M phosphate buffer pH 7 with 2 mg/ml haemoglobin in the temperature range from 55 to 72°. After the desired time, aliquots of the haemoglobin solution were rapidly chilled in an ice bath and centrifuged at 27,000 g for 15 min at 4°. The absorbance, A_t of the supernatant solution was measured at 523 nm (an isobestic point for HbO_2 and Hb^+). The ratio of the concentration of haemoglobin at time t = 0, $(Hb)_0$, to that at time t, $(Hb)_t$, was calculated with the following expression:

$$(Hb)_0/(Hb)_t = A_o/A_t$$

pH was checked before and after each experiment.

Urea denaturation rate measurements. Measurements of denaturation rate were carried out spectrophotometrically as previously described [22]. The reaction was followed at 578 nm for oxyhaemoglobin and 500 nm for ferrihaemoglobin. The urea concentration was 10 M for oxyhaemoglobin and 8 M for ferrihaemoglobin. The experiments were carried out in 0.1 M phosphate buffer with 2 mg/ml^{-1} haemoglobin at 20°. For each reaction, the mole fraction of unchanged haemoglobin at time t is represented by the equation:

$$(Hb)_t/(Hb)_0 = (A_t - A_\infty)/(A_0 - A_\infty),$$

In order to avoid ammonium cyanate formation, urea solutions were prepared from solid urea immediately before use. The pH was checked before and after each experiment.

RESULTS

Molecular weight dispersion

Molecular weight dispersion of cross-linked soluble polymers was shown by gel filtration on sepharose 6B (Fig. 1). The reaction of glutaraldehyde with oxyhaemoglobin produces polymers of which about 30% are eluted in the void volume of the gel (molecular weights greater than 4×10^6), whereas the effect of glutaraldehyde on deoxyhaemoglobin yields polymers of a smaller size.

Effect of glutaraldehyde on oxidation–reduction potentials

Potentials at 50% oxidation of native haemoglobin and haemoglobin cross-linked in oxy, deoxy and ferri states have been determined for different values of pH (Fig. 2). The values of $E_{\frac{1}{2}}$ for native haemoglobin are in agreement with those previously reported under similar conditions by Antonini *et al.* [23]. The oxidation–reduction potentials are reduced after immobilization. Polymers obtained by cross-linking oxyhaemoglobin and methaemoglobin exhibited a smaller $E_{\frac{1}{2}}$ than those prepared from the deoxyhaemoglobin. The position of the curves and the magnitude of the oxidation Bohr effect are not different from that of native haemoglobin ($-\Delta E/\Delta pH = 0.55$).

* Abbreviations: Hb, haemoglobin; HbO_2, oxyhaemoglobin; Hb^+, ferrihaemoglobin; $E_{\frac{1}{2}}$, potential at 50% oxidation.

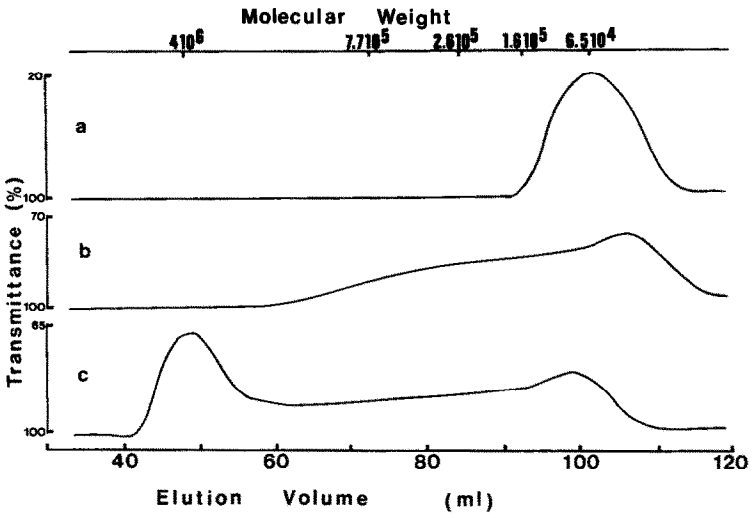

Fig. 1. Sepharose 6 B gel filtration of glutaraldehyde cross-linked haemoglobin. Native (a) and cross-linked haemoglobin in the deoxy state (b) and in the oxy state (c). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Gel filtration: bed volume 110 ml, 1 ml samples were eluted with 0.1 M phosphate buffer pH 6.8 at a flow rate of 9 ml/hr. Molecular weights markers: dextran blue, thyroglobulin, pyruvate kinase, aldolase and haemoglobin.

The shape of the oxidation–reduction equilibrium curves was estimated by n (linear regression from $Y = 0.15$ to $Y = 0.85$ on Hill plots of oxidation–reduction equilibria) for different pH (Fig. 3). Haemoglobin immobilization in the oxy or ferri state suppressed cooperativity whereas cooperativity of haemoglobin cross-linked in the deoxy-state is only reduced. Hill numbers smaller than 1 have been already measured for oxygen binding with the same preparation of cross-linked soluble polymers and have been explained by the heterogeneity of the polymers preparation [16].

Effect of glutaraldehyde on autoxidation kinetics

Autoxidation kinetics were followed in 0.1 M phosphate buffer from 10 to 55°. Kinetic and thermodynamic analysis was carried out with the initial rates of autoxidation. Thermodynamic parameters for the activation of autoxidation in the pH range 5.5–8 of native oxyhaemoglobin and haemoglobin cross-linked by glutaraldehyde in the oxy and deoxy states are summarized in Table 1. Autoxidation rates of cross-linked haemoglobin were up to four times greater than that of native haemoglobin.

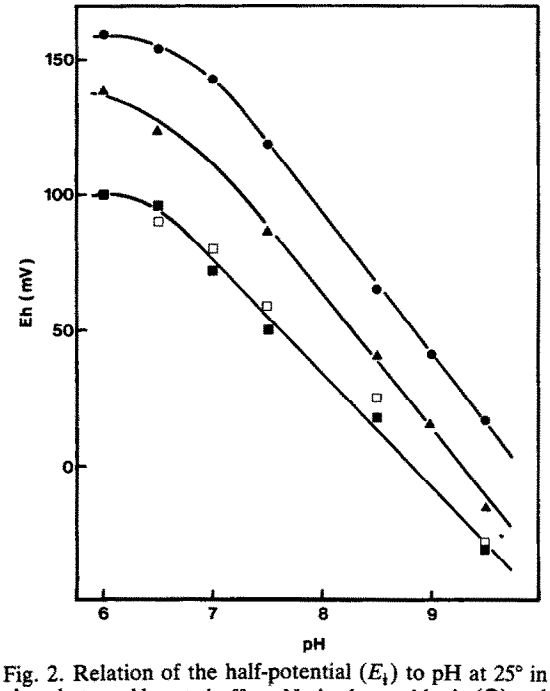

Fig. 2. Relation of the half-potential ($E_{\frac{1}{2}}$) to pH at 25° in phosphate and borate buffers. Native haemoblogin (●) and cross-linked in the oxy state (■) in the ferri state (□) and in the deoxy state (▲). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°.

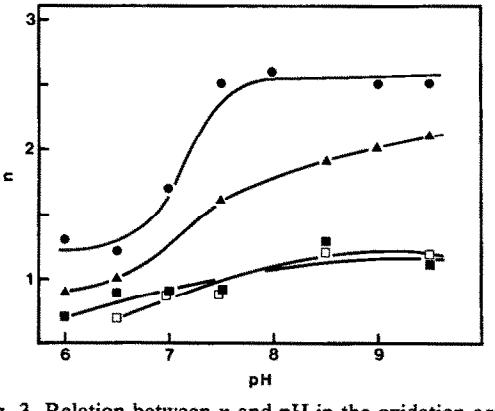

Fig. 3. Relation between n and pH in the oxidation equilibrium in 0.1 M phosphate and borate buffers at 25°. Native haemoglobin (●) and cross linked in the oxy state (■) in the ferri state (□) and in the deoxy state (▲). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°.

Table 1. Thermodynamic parameters for the activation of autoxidation of native haemoglobin and haemoglobin cross-linked in the oxy or deoxy states. Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Autoxidation kinetics: 0.1 M phosphate buffer, temperature range from 10 to 55°

Autoxidation pH	Native haemoglobin or cross-linked as	Ea Kcal/mol	$\Delta H_{25}^{\circ\neq}$ Kcal/mol	$\Delta S^{\circ\neq}$ Cal/mol K	$\Delta G_{25}^{\circ\neq}$ Kcal/mol
	Native haemoglobin	34.6	34.0	31	24.7
5.5	Oxy state	29.4	28.8	15	24.3
	Deoxy state	29.8	29.2	16	24.3
	Native haemoglobin	33.3	32.7	26	24.9
6.0	Oxy state	30.4	29.9	18	24.5
	Deoxy state	30.1	29.5	17	24.5
	Native haemoglobin	34.3	33.8	27	25.7
7.0	Oxy state	32.1	31.5	22	25.1
	Deoxy state	30.3	29.8	16	25.0
	Native haemoglobin	32.5	31.9	20	26.0
7.5	Oxy state	33.2	32.6	23	25.8
	Deoxy state	32.1	31.5	21	25.2
	Native haemoglobin	34.7	34.1	27	26.1
8.0	Oxy state	36.2	35.6	34	25.4
	Deoxy state	35.5	35.0	32	25.4

There is no significant difference between autoxidation rates of haemoglobin cross-linked in the oxy or in the deoxy state. The dependence of the rate of ferrihaemoglobin formation, at a given temperature, upon pH for native and immobilized haemoglobin is summarized in Fig. 4. Pseudo first order constants of cross-linked haemoglobin are less sensitive to pH than that of native haemoglobin. The autoxidation rate difference between native and cross-linked haemoglobin becomes greater at basic pH than at acidic pH, as the temperature is increased.

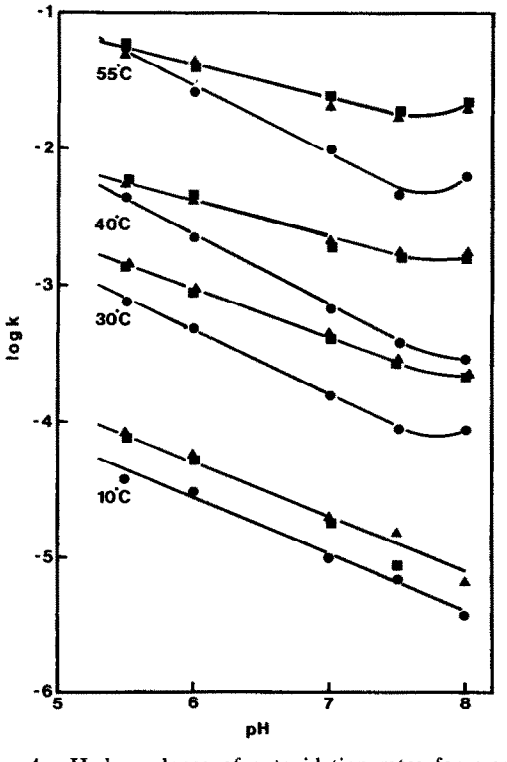

Fig. 4. pH dependence of autoxidation rates for cross-linked soluble polymers and native haemoglobin. Haemoglobin cross-linked in the oxy state (■) in the deoxy state (▲) and native haemoglobin (●). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Autoxidation kinetics: air-saturated 0.1 M phosphate buffer, at, from the bottom to the top, 10, 30, 40 and 55°.

Effect of glutaraldehyde on stability towards heat and urea

In order to appraise the stability of cross-linked haemoglobin towards denaturant agents, the effects of a physical agent (temperature) and of a chemical reagent (urea) were examined. The stability of cross-linked polymers towards heat was studied by following the production of insoluble protein as a function of time.

Thermodynamic parameters for activation determined at pH 7 for haemoglobin cross-linked in the oxy, deoxy and ferri state, and compared to native oxy and ferri haemoglobin are summarized in Table 2. As a general rule, changes in enthalpy and entropy of activation are decreased after cross-linking. This decrease in activation parameters is particularly important in the case of ferrihaemoglobin. The cross-linking by glutaraldehyde stabilizes haemoglobin at high temperature and destabilizes it at low temperature. So, the isokinetic temperatures for haemoglobin cross-linked in the oxy, deoxy and ferri state are respectively 58, 60 and 51°.

The pH dependence of the denaturation rate at 60° is summarized in Fig. 5 and is similar to that earlier reported by Lewis [24] and Levy *et al.* [25]. Whatever the ligation state and the oxidation state of the protein at the time of the cross-linking, the denaturation rates of cross-linked polymers are reduced compared to native oxy and ferri haemoglobin and are less sensitive to pH.

As for thermal denaturation, native and cross-linked ferrihaemoglobin are more sensitive to urea denaturation than native and cross-linked oxyhaemoglobin. The effect of crosslinking of haemoglobin upon pseudo first order constants of denaturation reactions is dependent on pH (Fig. 6).

Table 2. Thermodynamic parameters for the activation of thermal denaturation of native haemoglobin and cross-linked haemoglobin. Immobilizations: 0.5 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Denaturation kinetics: 0.1 M phosphate pH 7 buffer, temperature range from 55 to 72°

Native haemoglobin or cross-linked as	Ea Kcal/mol	$\Delta H^{\circ\neq}_{25}$ Kcal/mol	$\Delta S^{\circ\neq}$ Cal/mol/K	$\Delta G^{\circ\neq}_{25}$ Kcal/mol	$\Delta G^{\circ\neq}_{70}$ Kcal/mol
Native oxyhaemoglobin	74.2	73.6	153	28.0	21.1
Oxy state	61.3	60.7	114	26.8	21.6
Deoxy state	59.2	58.6	108	26.4	21.4
Native ferrihaemoglobin	85.4	84.8	190	28.0	18.8
Ferri state	48.1	47.5	75	25.1	21.7

Glutaraldehyde cross-linking stabilizes haemoglobin against urea denaturation only at acidic pH and when haemoglobin is cross-linked in the oxy state. On the other hand, whatever the pH ferrihaemoglobin is stabilized by glutaraldehyde cross-linking.

It was observed, as already shown for urea and alkali denaturation [22] that the spectra of urea denaturated haemoglobin and of thermally denaturated haemoglobin (precipitate solubilized in a mixture of sodium dodecyl sulfate and β mercaptoethanol) were identical to spectra of hemichromes (result not shown).

DISCUSSION

A detailed structural interpretation of the findings reported above is of course difficult, due to the lack of information on the number and the identity of the groups involved in the cross-linking, and to the heterogeneity of the cross-linked haemoglobin preparations. However, it is possible to draw observations regarding the effects of glutaraldehyde on the protein.

Heterogeneity of glutaraldehyde-cross-linked soluble polymers

The heterogeneity of cross-linked soluble polymers is not restricted to molecular weights (Fig. 1). We have previously shown [16] by electrophoresis that glutaraldehyde modified haemoglobin (without polymerization) is chemically heterogeneous and that the reactivity of glutaraldehyde is more important with the oxy form than with the deoxy form. This explains the higher degree of polymerization obtained with oxy- than with deoxyhaemoglobin. However, the biochemical properties of glutaraldehyde-cross-linked haemoglobin are rather related to the chemical modification of the protein

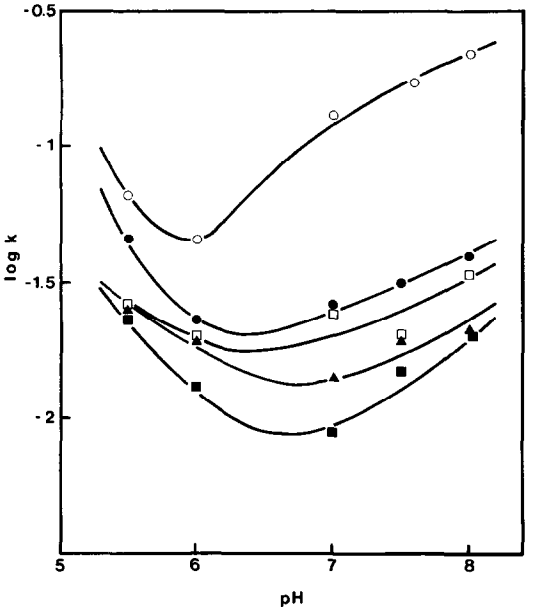

Fig. 5. pH dependence of thermal denaturation rates for cross-linked soluble polymers and native haemoglobin. Haemoglobin cross-linked in the oxy state (■) in the deoxy state (▲) in the ferri state (□) and native haemoglobin in the oxy (●) and in the ferri state (○). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Denaturation kinetics: 0.1 M phosphate buffer at 60°.

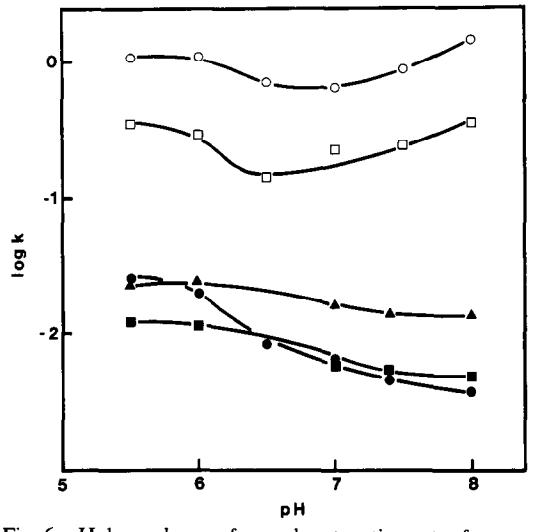

Fig. 6. pH dependence of urea denaturation rates for cross-linked soluble polymers and native haemoglobin. Haemoglobin cross-linked in the oxy state (■) in the deoxy state (▲) in the ferri state (□) and native haemoglobin in the oxy (●) and in the ferri state (○). Immobilizations: 0.05 M phosphate buffer, pH 7.2, containing 100 mg/ml haemoglobin, 3.3 mg/ml glutaraldehyde at 20°. Denaturation kinetics: 0.1 M phosphate buffer at 20°, urea concentration: 10 M (oxyhaemoglobin) and 8 M (ferrihaemoglobin). The pseudo first order constants measured correspond to initial velocities.

than to its polymerization degree since oxygen affinities of glutaraldehyde modified haemoglobin (without polymerization), cross-linked soluble polymers and insoluble polymers are not very different.

Effect of glutaraldehyde upon oxidation–reduction potentials

Whatever the oxidation and the ligation state of haemoglobin at the time of glutaraldehyde cross-linking, we have observed a decrease of oxidation–reduction potentials, an important decrease of cooperativity and an unchanged oxidation Bohr effect. These results for oxidation–reduction equilibria can be compared to those obtained for oxygen equilibria of glutaraldehyde-cross-linked haemoglobin [16] for which we have shown an increase of oxygen affinity, a suppression of cooperativity and an unchanged Bohr effect.

A correlation between an increase in oxygen affinity and a decrease in redox potential has been already shown with chemically modified haemoglobin by several authors [17, 26]; the mechanisms that control the oxidation–reduction equilibria would be the same as those that control the oxygen equilibria and may be correlated to the similarity of the conformation of ferrihaemoglobin and of oxyhaemoglobin [27]. Furthermore the modification of redox potential, as for oxygen affinity, is greater with increasing reaction of glutaraldehyde with the haemoglobin. So, the change in oxidation–reduction potential of haemoglobin cross-linked in the deoxy state, compared to native haemoglobin, is smaller than for haemoglobin cross-linked in the oxy or ferri state. We have already shown that the modification of haemoglobin with glutaraldehyde was greater when dealing with oxyhaemoglobin than when dealing with deoxyhaemoglobin. On the other hand the decrease in the redox potential of haemoproteins has already been correlated with the exposure of the haem to the aqueous solvent [28].

Homotropic interactions in oxidation–reduction equilibria of cross-linked haemoglobin are reduced, particularly in the case of cross-linked oxy or ferrihaemoglobin for which chemical modifications are more significant. However, contrary to the situation for oxygen equilibria, homotropic interactions are not totally suppressed. This seems to indicate different mechanisms of haem–haem, interactions for oxygen equilibria and for oxidation–reduction equilibria.

Effect of glutaraldehyde upon autoxidation

Whatever the pH, glutaraldehyde cross-linking increases the autoxidation rates of oxyhaemoglobin, and haemoglobin cross-linked in the oxy or deoxy state is oxidized at the same rate. It has been shown that aminoacid substitution in the protein does influence the susceptibility of the haemoglobin to autoxidation [20, 29]. Autoxidation rate differences correlate well with steric hindrance in the haem pocket; as steric hindrance decreases, the rate of autoxidation increases. Moreover studies on model compounds show that a decrease in steric hindrance lowers the oxidation potential and increases oxygen affinity [30]. For cross-linked haemoglobin, decrease of the redox potential, increase of oxygen affinity and increase of autoxidation rates could be explained by an opening of the haem pocket after the glutaraldehyde cross-linking.

The dependence of the rate of autoxidation upon pH is reduced for cross-linked haemoglobin compared to native haemoglobin. The groups involved in the effect of pH on autoxidation rates for native haemoglobin are not identified. The linear dependence of the rate upon (H^+) was explained by Wallace *et al.* [20] as a specific acid catalysis which involves the protonation of a site on the protein with a pka below 5. The autoxidation rate at atmospheric pO_2 would be: Rate = $k1$ (Nucleophile) (HbO_2) $(H^+)/Ka + (H^+) + k2$ (Nucleophile) (HbO_2) $Ka/Ka + (H^+)$ with $k1 \gg k2$.

The decrease in the slopes after glutaraldehyde cross-linking, whatever the temperature (Fig. 4) could be explained by an increase in the pka of the group involved in the acidic catalysis. However as the temperature increases the slope decreases. This could be explained by an increase in the $k2$ constant (autoxidation rate constant of the unprotonated form of oxyhaemoglobin) with increased temperature. This effect would be greater at basic pH. In fact at basic pH the value of the pseudo first order constant, compared to native haemoglobin, is controlled by the activation entropy change (Table 1), whereas at acidic pH the decisive factor is activation enthalpy change. The modification of the dependence of the rate of autoxidation upon pH, after cross-linking, could be also explained by a deformation or an opening of the haem pocket which would increase the accessibility of the nucleophiles to the iron.

Effect of glutaraldehyde on stability

The stability of cross-linked haemoglobin, compared to native haemoglobin, depends greatly on the nature of the denaturing agent, on the temperature range and on the pH. Whatever the denaturation pH and whatever the ligation state and the oxidation state of haemoglobin at the time of immobilization, glutaraldehyde stabilizes haemoglobin against thermal denaturation at high temperature. Oxyhaemoglobin is better stabilized when immobilized in the oxy state than in the deoxy state; this is in agreement with the fact that glutaraldehyde reacts better with oxyhaemoglobin than with deoxyhaemoglobin as we have previously shown [16]. However the glutaraldehyde cross-linking stabilizes ferrihaemoglobin better than oxyhaemoglobin in spite of a similarity in structure [27]. The decisive factor in the stabilization at high temperature by cross-linking is the decrease in entropy of activation (Table 2). Native ferrihaemoglobin has a greater entropy of activation than native oxyhaemoglobin; so the stabilization by glutaraldehyde cross-linking is more efficient for ferrihaemoglobin than for oxyhaemoglobin. The pH dependence of the denaturation rates for native haemoglobin was interpreted by Levy and Benaglia [25]. Glutaraldehyde cross-linking does not change the shape of the curve to a great extent but probably alters the pka of the groups involved in the denaturation process because the sensitivity of denaturation rates to pH is reduced.

Urea denaturation of haemoglobin is not as well understood as thermal denaturation. Urea acts as an

excellent hydrogen-bonding agent and at least in part by altering the solvation of exposed hydrophobic side chains in the denatured state [31]. In fact gluraraldehyde cross-linking stabilizes only ferrihaemoglobin against urea denaturation whatever the pH. The denaturation scheme generally accepted for oxyhaemoglobin is: Oxyhaemoglobin → Ferrihaemoglobin → Hemichromes → precipitates [32, 33].

Glutaraldehyde stabilizes in particular ferrihaemoglobin for which the unfolding of the protein is the more important (activation entropy changes are greater). The first stage of the denaturation which involves movements of protein chains smaller than those of the second stage has its rate reduced less by cross-linking than the rate of the second stage.

The fact that glutaraldehyde cross-linking does not reduce the rate of the first stage could also be explained by an opening of the haem pocket, after cross-linking, which would facilitate the action of urea.

In conclusion, our results show that the impact of glutaraldehyde on haemoglobin involves significant modifications of behaviour which must be taken into account in the design of a blood substitute or for technological purposes.

These modifications can be summarized as follows:

(i) The autoxidation rates of cross-linked oxyhaemoglobin are about four times higher than that of native oxyhaemoglobin at physiological temperature and pH.

(ii) The cross-linking stabilizes haemoglobin against thermal denaturation at high temperatures and destabilizes haemoglobin at physiological temperature.

(iii) Glutaraldehyde cross-linking only hinders conformation changes of great amplitude. The effects of glutaraldehyde on oxidation–reduction equilibria, autoxidation kinetics and denaturation kinetics, as already shown in an earlier paper [16] for oxygen binding equilibria, can mainly be imputed to the chemical modification of the protein, and not to the freezing of conformations.

(iv) Glutaraldehyde cross-linking probably involves the opening of the haem pocket to the solvent. Mossbauer spectral studies on glutaraldehyde modified haemoglobin are undertaken to confirm this hypothesis.

REFERENCES

1. S. A. Gould, A. L. Rosen, L. Sehgal, H. Sehgal, C. L. Rice and G. S. Moss, *Trans. Am. Soc. Artif. Intern. Organs* **26**, 350 (1980).
2. F. De Venuto and A. Zegna, *Surg. Gynecol. Obstet.* **155**, 342 (1982).
3. P. J. Scannon, *Crit. Care Med.* **10**, 261 (1982).
4. L. R. Sehgal, A. L. Rosen, S. A. Gould, H. L. Sehgal and G. S. Moss, *Transfusion* **23**, 158 (1983).
5. P. E. Keipert and T. M. S. Chang, *Trans. Am. Soc. Artif. Intern. Organs* **24**, 329 (1983).
6. L. R. Sehgal, S. A. Gould, A. L. Rosen and G. S. Moss, *Surgery* **95**, 433 (1984).
7. M. Feola, H. Gonzalez, P. C. Canizaro, D. Bingham and P. Periman *Surg. Gynecol. Obstet.* **157**, 399 (1983).
8. F. M. Richards and J. R. Knowles *J. molec. Biol.* **37**, 231 (1968).
9. P. M. Hardy, A. C. Nicholls and H. N. Rydon, *Chem. Commun.* 565 (1969).
10. A. H. Korn, S. H. Feairheller and E. M. Filachione, *J. molec. Biol.* **65**, 525 (1972).
11. J. W. Payne, *Biochem. J.* **135**, 867 (1973).
12. P. Monsan, G. Puzo and H. Mazarguil, *Biochimie* **57**, 1281 (1975).
13. K. Peters and F. M. Richards, *A. Rev. Biochem.* **46**, 523 (1977).
14. S. Branner-Jorgensen, *Enzyme Eng.* **4**, 393 (1978).
15. D. T. Cheung and M. E. Nimmi, *Connect. Tissue Res.* **10**, 187 (1982).
16. D. Guillochon, L. Esclade, M. H. Remy and D. Thomas, *Biochim. biophys. Acta* **670**, 332 (1981).
17. E. Antonini and M. Brunori, *Hemoglobin and Myoglobin in their Reactions with Ligands*, Vol. 21, North-Holland, Amsterdam (1971).
18. D. Drabkin, *Am. J. Med. Sci.* **217**, 710 (1949).
19. W. M. Clark, *Oxidation–Reduction Potentials of Organic Systems*. The Williams and Wilkins Company, Baltimore (1960).
20. W. J. Wallace, R. A. Houtchens, J. C. Maxwell and W. S. Caughey, *J. biol. Chem.* **257**, 9, 4966 (1982).
21. A. Cupane, D. Giacomazza and L. Cordone, *Biopolymers* **21**, 1081 (1982).
22. F. D. White, A. Kerr, R. B. Ferguson and A. R. Hull, *Can. J. Biochem.* **38**, 443 (1960).
23. E. Antonini, J. Wyman, M. Brunori, J. F. Taylor, A. Rossi-Fanelli and A. Caputo, *J. biol. Chem.* **239**, 907 (1964).
24. P. S. Lewis, *Biochem. J.* **20**, 965 (1926).
25. M. Levy and A. E. Benaglia, *J. biol. Chem.* **186**, 829 (1950).
26. S. J. Edelstein and Q. Gibson, *J. biol. Chem.* **250**, 961 (1975).
27. B. Shaanan, *J. molec. Biol.* **171**, 31 (1983).
28. E. Stellwagen *Nature, Lond.* **275**, 73 (1978).
29. W. J. Wallace, R. A. Houtchens, J. M. Holt and W. S. Caughey, *Biochemical and Clinical Aspects of Haemoglobin Abnormalities*, pp. 477–493 Academic Press, New York (1978).
30. T. G. Traylor and P. S. Traylor, *A. Rev. Biophys. Bioengng* **11**, 105 (1982).
31. T. T. Herskovits and H. Jaillet, *Science, Wash.* **163**, 282 (1969).
32. E. A. Rachmilewitz, J. Peisach and W. E. Blumberg, *J. biol. Chem.* **246**, 3356 (1971).
33. T. C. Hollocher, *J. biol. Chem.* **241**, 1958 (1966).