Salt Effect on Acid-Catalyzed Autoxidation of Oxymyoglobin

H. J. Andersen, G. Bertelsen and L. H. Skibsted

Chemistry Department, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Andersen, H. J., Bertelsen, G. and Skibsted, L. H., 1988. Salt Effect on Acid-Catalyzed Autoxidation of Oxymyoglobin. – Acta Chem. Scand., Ser. A 42: 226–236.

The rate of the specific acid-catalyzed autoxidation of equine oxymyoglobin increases in aqueous sodium chloride solution of pH between 5 and 6 with increasing ionic strength, the increase corresponding to that expected for a reaction between a proton and a reactant with an effective positive charge of one. In 0.16 M sodium chloride the activation parameters are $\Delta H^{\ddagger}_{298}$ = 117(2) kJ mol^{-1} and $\Delta S^{\ddagger}_{298}$ = 172(5) J mol^{-1} K^{-1}. In aqueous sodium perchlorate, the ionic strength dependence is that expected for a reaction between a proton and a reactant with an effective positive charge of two, rendering the acid-catalyzed autoxidation in 0.16 M sodium perchlorate ca. 3 times as fast as in 0.16 M sodium chloride. However, the enthalpy of activation is the same in the two salt media, and the difference in rate of autoxidation between the two media vanishes gradually for both equine- and bovine oxymyoglobin when pH is increased and the uncatalyzed autoxidation becomes the dominating reaction path. These observations are discussed in relation to anion binding and salt denaturation of myoglobins.

The decrease in pH that accompanies postmortem glycolysis in meat accelerates the autoxidation of oxymyoglobin (MbO_2) to metmyoglobin (MMb) as this latter process is subject to acid catalysis.[1] The autoxidation of oxymyoglobin has been found to give the superoxide ion as a second reaction product,[2,3] and this highly reactive radical-ion is a potential initiator for chain reactions such as those leading to deterioration of lipids and development of rancidity.[4]

Discoloration of fresh and frozen meat is primarily a result of either thermal or photochemical oxidation of the bright red oxymyoglobin to the brown metmyoglobin,[5] and such discoloration might thus serve as an indicator of oxidative rancidity. Other factors beside temperature, light and pH are suspected as influencing the primary autoxidation of oxymyoglobin, and since it is common practice to add sodium chloride to certain ground beef products, and since such products pose colour-stability problems,[6] we have undertaken an investigation of the effect of ionic strength on the reaction rate in the pH region relevant for meat and meat products. In order to separate a primary salt effect from any specific effect of the nucleophilic chloride ion on the autoxidation, we have included experiments in which the ionic strength is adjusted with sodium trifluoromethanesulfonate or sodium perchlorate. The results of these investigations form the basis for a comment on proposed mechanisms[2,3,7–9] for oxymyoglobin autoxidation, whereas more practical aspects of the kinetic salt effect will be commented on elsewhere.[6]

Experimental

Bovine oxymyoglobin. Myoglobin was extracted from bovine hearts and fractionated at 0–4 °C according to the method, slightly modified, of Gotoh and Shikama.[10] A slurry of minced fresh bovine hearts and 1.5 volumes of deionized water was adjusted to pH = 8.00(5) and extracted overnight. The extract was fractionated with ammonium sulfate solution of between 60 and 100 % saturation at pH = 6.80(5) in the presence of 0.5 mM EDTA. After centrifugation (14,000 g for 30 min), the precipitate was dialyzed overnight

against a 5 mM Tris,HCl buffer with pH = 8.40(5) containing 0.5 mM EDTA. The crude myoglobin solution was applied to Sephadex G-50 columns (5×60 cm) which had been equilibrated with the buffer used for the dialysis and were subsequently eluted with the same buffer to separate myoglobin completely from hemoglobin. The effluent myoglobin solution was concentrated by a factor of 3–4 by ultrafiltration (10 PM10 filter) and dialyzed against the same pH = 8.40 buffer. The dialyzed myoglobin solution was applied to Fractogel TSK DEAE-650 columns (2.6×18 cm) which had been equilibrated with the pH = 8.40 buffer, and the columns were washed with 15 mM Tris,HCl buffer with pH = 8.00(5) to elute the brown-coloured band of metmyoglobin. Oxymyoglobin was subsequently eluted with 30 mM Tris,HCl buffer with pH = 8.00(5). Oxymyoglobin solutions were stored at 0–4 °C and concentrated by ultrafiltration when required for use.

Equine oxymyoglobin. Metmyoglobin (Sigma Type III) was dissolved in doubly-deionized water (0.1 g ml^{-1}) and purified on a Sephadex G-50 column by elution with a 5 mM equimolar $H_2PO_4^-/HPO_4^{2-}$ buffer. MMb was converted to MbO_2 by reduction with $Na_2S_2O_4 \cdot xH_2O$ (Merck, 10 mg per 100 mg metmyoglobin), and the product solution was subsequently passed through a mixed-bed ion exchanger at 0 °C (Dowex 50W-X8, 100–200 mesh, plus Dowex 1 X8, 20–50 mesh).

Other materials. Imidazole used for the determination of pK_a for the imidazolium ion was obtained from Fluka (*puriss. p.a.*). Trifluoromethanesulfonic acid from the 3 M Company was neutralized with sodium hydrogen carbonate, and $NaCF_3SO_2O$ was purified by fractional crystallization. Other chemicals were of analytical grade. The reaction media were analyzed using standard methods.

Buffers and pH measurement. Acetate, phthalate, phosphate and tris(hydroxymethyl)aminomethane buffers were used for the kinetic experiments, and the ionic strength was adjusted with the appropriate salt. For the determination of the pK_a of coordinated water in metmyoglobin, borax and carbonate buffers were also used. pH was in each case measured relative to *concentration* standards in the actual salt medium (titrated solutions of strong acid), and the definition pH = $-\log[H^+]$ was employed for all kinetic and equilibrium measurements. It should be noted, however, that the buffer solution used for protein separation and the protein denaturation studies was adjusted relative to the international pH standards. A Metrohm 605 pH meter and combination glass electrodes (Ingold type 405) were used for pH measurement, with either 3 M KCl or, when used in a perchlorate medium, 1.0 M NaCl in the reference part.

Determination of pK_a values. Imidazolium ion: Weighed amounts of imidazole (ca. 0.2 mmol) were dissolved in 25.00 ml of a standardized 10^{-2} M HCl, 0.15 M NaCl solution (or other salt medium of the same ionic strength) and titrated to pH = 11 with 0.05 M NaOH in the actual salt medium. pK_a under each set of conditions was calculated from two full titration curves as previously described.[11] *MMb:* Solutions of purified equine MMb were mixed with buffer solutions to give an appropriate absorbance and a total buffer concentration of 0.020 M, and the ionic strength was adjusted with the sodium salts listed in Table 4. pH was measured and the 450 to 700 nm absorption spectrum was recorded immediately and again after 2 h (Zeiss DMR 21 spectrophotometer). For each set of conditions, three to six different buffers with pH values corresponding to a degree of MMb neutralization ranging between 0.2 and 0.8 were used, and pK_a values were calculated from the absorbance at 540, 549, 580 and 591 nm using standard methods.[12] For each salt medium, the absorption spectrum of the acidic and the basic form of MMb (both required for the calculations) were recorded at pH = 6 and at pH = 11.5, respectively.

Protein denaturation. Pre-thermostatted solution of purified equine MMb were mixed with standardized salt solutions to give a series of increasing salt concentrations, and so that the absorbance of the Soret band (λ_{max} = 408 nm) was close to unity in the more dilute salt solutions. Absorption spectra ($350 < \lambda < 450$ nm) were recorded after $^1/_2$ h and 4 h, during which time interval only minor changes were noted.

Kinetic experiments. Pre-thermostatted solutions of equine or bovine MbO_2 were mixed with

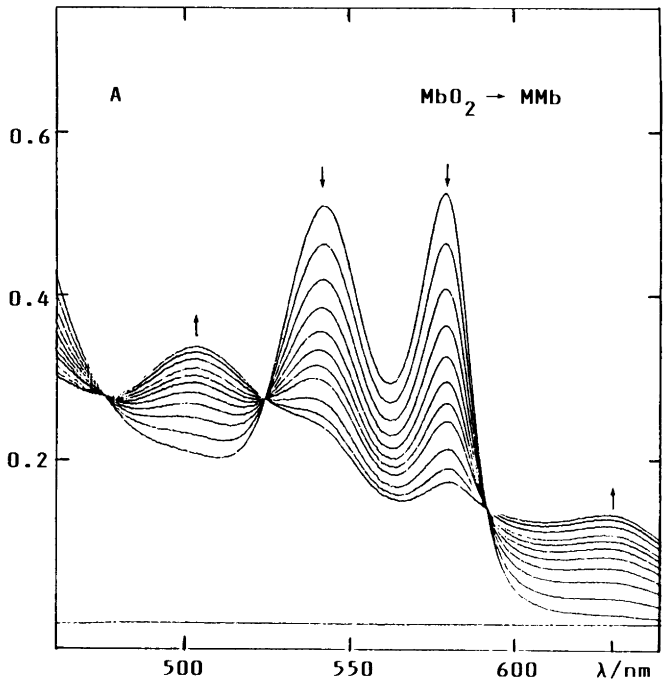

Fig. 1. Spectral changes during autoxidation of bovine oxymyoglobin at 30.0 °C. An MbO_2 solution ($c_{MbO_2} = 3.60 \cdot 10^{-5}$ M as calculated from absorption data)[10] in a pH = 5.03 acetate buffer (0.020 M) with ionic strength 0.16 adjusted with NaCl was scanned after temperature equilibration and subsequently after 10, 20, 30, 40, 50, 60, 70, 90, 110 and 130 min. Calculation of first-order rate constants according to $A_\lambda(t) = a_\lambda + b_\lambda \cdot e^{-k_{obs}t}$ gave $k_{obs} = 3.01(2) \cdot 10^{-4}\ s^{-1}$, indicating that the reaction was followed for 3.4 halflives. The quantity *var* divided by *f* (degrees of freedom) has an expectation value close to unity and was found, for the present experiment, to have the value 0.85, showing consistency between model (one first-order reaction) and experiment (goodness of fit).

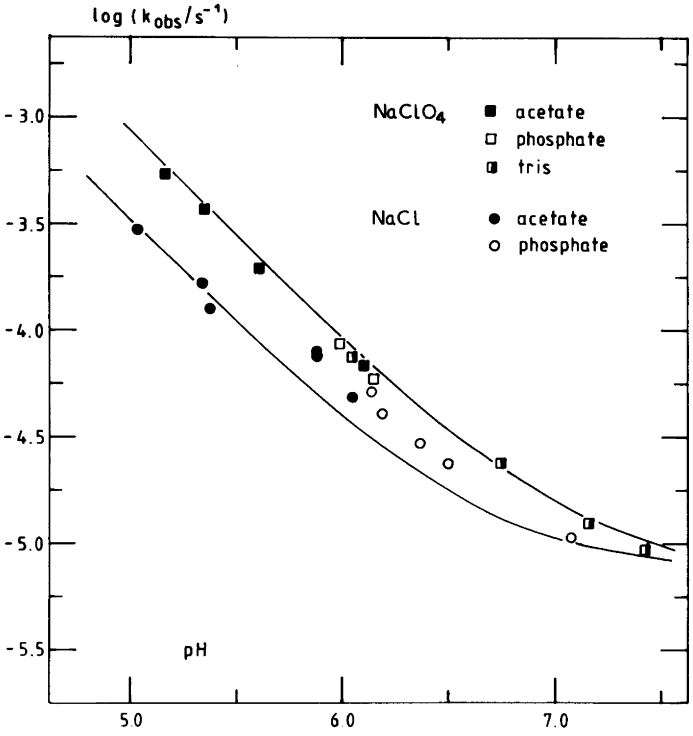

Fig. 2. Hydrogen-ion catalyzed autoxidation of bovine oxymyoglobin at 30.0 °C in aqueous solution of ionic strength 0.16 adjusted with sodium perchlorate (squares) or sodium chloride (circles). Logarithmic plot of observed first-order rate constants as a function of hydrogen-ion concentration according to $k_{obs} = k_0 + k_{H^+}^{X^-}[H^+]$, where $k_0 = 7.4(1.1) \cdot 10^{-6}\ s^{-1}$ is the first-order rate constant for a common (anion-independent), uncatalyzed autoxidation and $k_{H^+}^{Cl^-} = 33(2)\ l\ mol^{-1}\ s^{-1}$, $k_{H^+}^{ClO_4^-} = 84.1(1.1)\ l\ mol^{-1}\ s^{-1}$ are second-order rate constants for the hydrogen-ion catalyzed autoxidation in the chloride and perchlorate media, respectively. Note that $pH = -\log[H^+]$.

buffer solutions to give an appropriate absorbance and a total buffer concentration of 0.020 M, and the ionic strength was adjusted with the sodium salts listed in Table 2. The reaction mixture was transferred to 1 cm silica cells and placed in the thermostatted cell compartment of a Cary 219 spectrophotometer. Spectra were recorded at known times ($450 < \lambda < 650$ nm). The absorbance values in recorded spectra were read off at the four wavelengths 502, 542, 580 and 630 nm.

Calculations. Calculations were performed within the framework of non-linear regression analysis using a least-squares criterion for minimization. For the kinetic experiments, the different wavelengths were included in the same calculation,[13] and the quantity to be minimized was:

$$\text{var} = \sum_{\lambda} \sum_{t} \frac{[A_{\lambda,t}(\text{obs}) - A_{\lambda,t}(\text{calc})]^2}{\sigma_A^2} .$$

Results

Visible spectra, in combination with literature spectral data,[5,10,14] were used for identification of the purified pigments as oxymyoglobin and of the product of the autoxidation as metmyoglobin:

$$4\text{MbO}_2 + 4\text{H}^+ \rightarrow 4\text{MMb} + 3\text{O}_2 + 2\text{H}_2\text{O}. \quad (1)$$

The autoxidation involves a change in oxidation state from Fe(II) to Fe(III) of the chromophore responsible for the visible light absorption, and was easily monitored by direct spectrophoto-

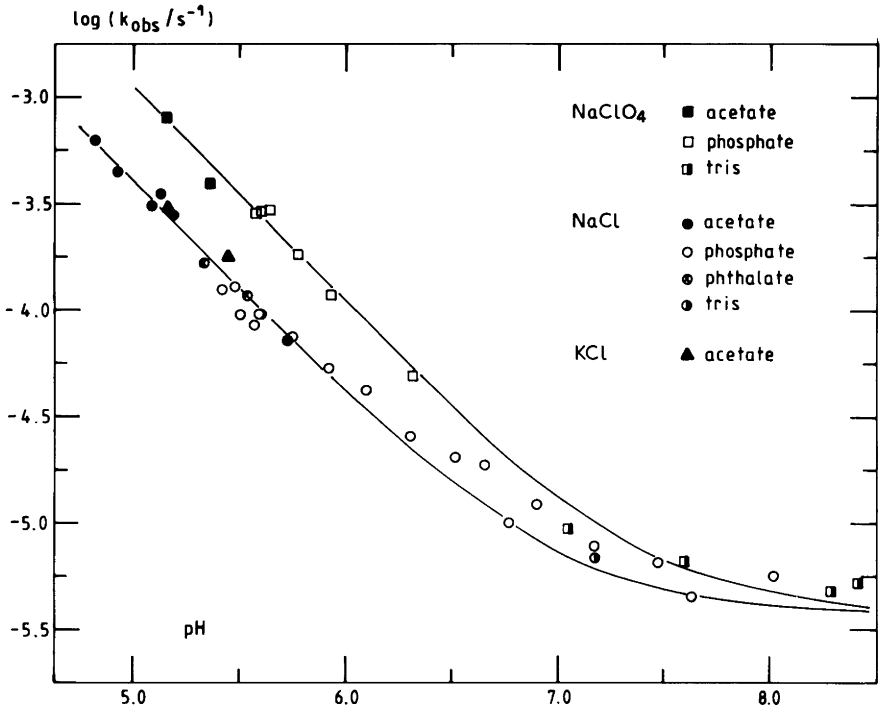

Fig. 3. Hydrogen-ion catalyzed autoxidation of equine oxymyoglobin at 30.0 °C in aqueous solution of ionic strength 0.16 adjusted with sodium perchlorate (squares), sodium chloride (circles) or potassium chloride (triangles). Logarithmic plot of observed first-order rate constants as a function of hydrogen-ion concentration according to $k_{obs} = k_0 + k_{H^+}^{X^-}[H^+]$, where $k_0 = 3.7(1.7) \cdot 10^{-6}$ s^{-1} is the first-order rate constant for a common (anion-independent), uncatalyzed autoxidation and $k_{H^+}^{Cl^-} = 46(2)$ and $k_{H^+}^{ClO_4^-} = 118(4)$ / mol^{-1} s^{-1} are second-order rate constants for the hydrogen-ion catalyzed autoxidation in the chloride and perchlorate media, respectively. Note that $pH = -\log[H^+]$.

Table 1. First-order rate constant for autoxidation of equine oxymyoglobin at 30.0 °C in aqueous solution with different buffer concentrations. Ionic strength 0.16 adjusted with sodium chloride.

c^a_{BUFFER}	Buffer	pH[b]	k_{obs}/s^{-1}
0.020	Acetate[c]	5.82	$7.45(10)\cdot10^{-5}$
0.040	Acetate[c]	5.80	$8.17(5)\cdot10^{-5}$
0.060	Acetate[c]	5.81	$8.37(5)\cdot10^{-5}$
0.010	Phosphate	6.39	$2.45(10)\cdot10^{-5}$
0.020	Phosphate	6.37	$2.30(10)\cdot10^{-5}$
0.030	Phosphate	6.35	$2.35(10)\cdot10^{-5}$
0.040	Phosphate	6.34	$2.03(10)\cdot10^{-5}$
0.060	Phosphate	6.34	$2.33(10)\cdot10^{-5}$

[a]Total buffer concentration. [b]pH = $-\log[H^+]$, measured in each case. [c]Linear regression according to $k_{obs} = k' + k_{CH_3COOH}[CH_3COOH]$ gave $k' = 7.3(4)\cdot10^{-5}\ s^{-1}$ and $k_{CH_3COOH} = 5(2)\cdot10^{-3}\ l\ mol^{-1}\ s^{-1}$.

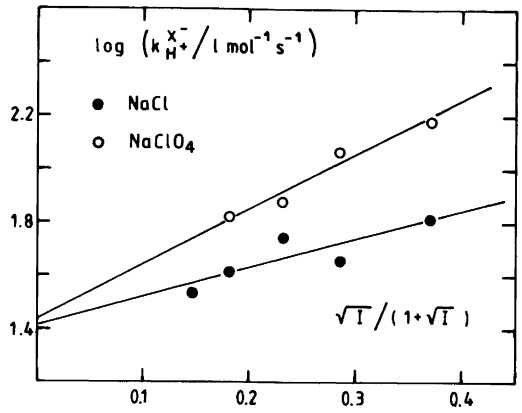

Fig. 4. The ionic strength dependence of the second-order rate constants for the hydrogen-ion catalyzed autoxidation of equine oxymyoglobin in aqueous solution at 30.0 °C plotted according to $\log k^{X^-}_{H^+} = \log k^0_{H^+} + 2\cdot0.51\cdot z_+\cdot z_{MbO_2}\cdot(\sqrt{I}/(1+\sqrt{I}))$. The slope, $2\cdot0.51\cdot z_+\cdot z_{MbO_2}$, depends on the anion of the medium and is +2.0±0.1 for sodium perchlorate and +1.0±0.1 for sodium chloride, whereas the intercept, $\log k^0_{H^+}$, is found to be independent of the anion present: 1.42±0.02 in the chloride and 1.44±0.02 in the perchlorate medium, respectively.

metry (see Fig. 1). In aqueous solution in equilibrium with the atmosphere, the autoxidation of both bovine and equine MbO_2 was found to follow first-order kinetics as tested by a chi-square criterion (Fig. 1) and in agreement with what has been found for MbO_2 of various origin.[9,10,15,16] The first-order rate constant is strongly dependent on solution pH, as seen in Fig. 2 and Fig. 3 for bovine and equine MbO_2, respectively, and also on the nature of the anion of the electrolyte used to adjust the ionic strength. For conditions of fixed pH, the rate depends only slightly on the nature of the buffer, as seen from Figs. 2 and 3. The variation with buffer concentration was tested for two of the buffer substances used and, as may be seen from Table 1, the rate constant does not depend on the phosphate concentration ($0.01 < c_{phosphate} < 0.06$ M), whereas a slight increase with increasing total acetate concentration was noted.

The linear dependence of the logarithm of the observed first-order rate constant on solution pH in the investigated salt media from pH ~ 5 (MbO_2 precipitates in more acidic solution) up to pH ~ 6 (the latter limit depends on the nature of the supporting electrolyte; Figs. 2 and 3) is con-

Table 2. Second-order rate constant for hydrogen-ion catalyzed autoxidation of equine oxymyoglobin at 30.0 °C in different aqueous salt media.[a]

Ionic strength[b]	$k^{Cl^-}_{H^+}$ // $l\ mol^{-1}\ s^{-1}$	$k^{Br^-}_{H^+}$ // $l\ mol^{-1}\ s^{-1}$	$k^{CF_3SO_2O^-}_{H^+}$ // $l\ mol^{-1}\ s^{-1}$	$k^{ClO_4^-}_{H^+}$ // $l\ mol^{-1}\ s^{-1}$
0.35	66(3)			152(3)
0.16	46(2)	62(2)	91(8)	118(4)
0.09	55(2)			74(7)
0.05	41(7)			66(3)
0.03	35(4)			

[a]Calculated as outlined in Fig. 2 for NaCl and $NaClO_4$ solutions of ionic strength 0.16. [b]Solution pH between 5 and 6. Buffer concentration 0.020 M.

sistent with an autoxidation mechanism involving specific acid catalysis,[7,8] as is the absence of influence of buffer concentration on the rate constant. From the dependence of the observed rate constant on hydrogen-ion concentration, the second-order rate constant for specific acid catalysis, $k_{H^+}^{X^-}$, was calculated for each of the salt media according to eqn. (2), in which k_0 is the first-

$$k_{obs} = k_{H^+}^{X^-}[H^+] + k_0 \qquad (2)$$

order rate constant for an uncatalyzed autoxidation. For both bovine and equine MbO_2, the model with two reaction paths corresponding to eqn. (2) fully describes the results obtained for the perchlorate medium (see Figs. 2 and 3), whereas for the chloride medium significant deviations are observed at intermediate pH. It should be noted, however, that the value obtained for k_0 in the perchlorate medium also accounts for the observed rate in neutral chloride solutions. The difference in rate of autoxidation noted for the perchlorate and chloride media vanishes in neutral solution (and similar results were obtained for bromide and trifluoromethanesulfonate), suggesting that the uncatalyzed autoxidation is anion-independent. From the results presented in Table 2 for the acid-catalyzed autoxidation at 30 °C, it is seen that in solutions of physiological ionic strength, the rate depends on the nature of the anion according to $ClO_4^- > CF_3SO_2O^- > Br^- > Cl^-$. This result was obtained for equine MbO_2 but is believed to apply also for bovine MbO_2, since the ratio $k_{H^+}^{ClO_4^-}/k_{H^+}^{Cl^-}$ was found to have the common value of 2.6(5) for the two different types of MbO_2. For the chloride and perchlorate media, the dependence of the second-order rate constant on the salt concentration is that expected for a reaction between a proton and a mono- and divalent cation, respectively, as may be seen from Fig. 4, in which the ionic strength dependence is analyzed according to the Debye-Hückel theory. A common rate constant valid as infinite dilution is obtained by extrapolation in the two media; in agreement herewith, the enthalpy of activation $\Delta H_{298}^{\ddagger} = 117(2)$ kJ mol^{-1} obtained for acid-catalyzed autoxidation in 0.16 M NaCl also accounts for the temperature dependence of this process in 0.16 M $NaClO_4$, as may be seen from Fig. 5.

Transfer of a proton to the imidazole ring of a histidyl residue in the heme pocket may play a key role in the acid-catalyzed autoxidation of oxymyoglobin,[8,9] and the thermodynamics of this process were characterized by titration of the imidazolium ion at different temperatures (Fig. 6). The values derived for $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ for the dissociation of a proton from the imidazolium ion

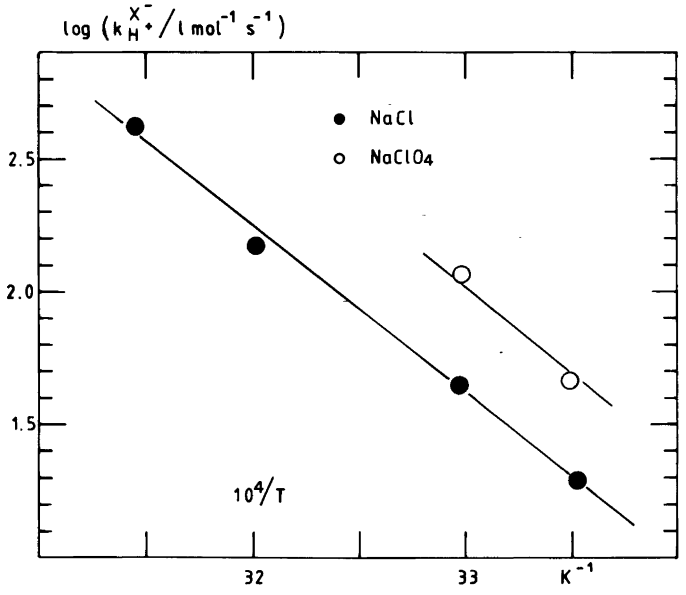

Fig. 5. The temperature dependence of the hydrogen-ion catalyzed autoxidation of equine oxymyoglobin in 0.16 M NaCl is well described by the Arrhenius equation. The activation parameters, calculated according to transition state theory, are $\Delta H_{298}^{\ddagger} = 117(2)$ kJ mol^{-1} and $\Delta S_{298}^{\ddagger} = 172(5)$ J mol^{-1} K^{-1}. The latter enthalpy of activation also accounts for the observed temperature dependence of the hydrogen-ion catalyzed autoxidation in 0.16 M $NaClO_4$.

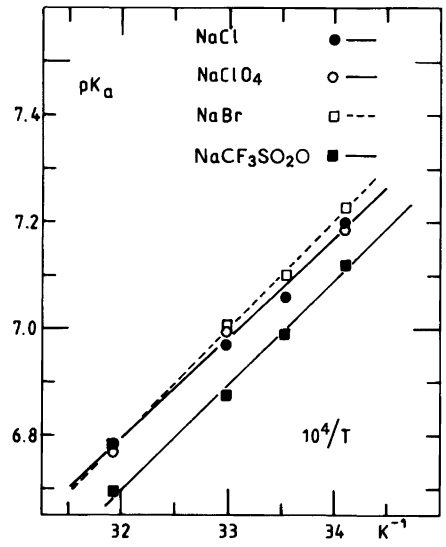

Fig. 6. Temperature dependence of pK_a for the imidazolium ion in 0.16 M solutions of different sodium salts. Experimental values were determined potentiometrically, and the lines computed from $pK_a = (\Delta H^{\ominus}/T - \Delta S^{\ominus})/2.30\ R$ are the basis for the parameters $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ for the acid dissociation (Table 3).

(and presented in Table 3) depend very little on the nature of the anion in the 0.16 M sodium salt media used in the present kinetic investigation.

Other extrakinetic probes of the influence of the salt media on the heme pocket (and on the acid-catalyzed autoxidation) are the acidity of the water coordinated to Fe(III) in the oxidation product MMb and the degree of denaturation of MMb caused by the salt. The denaturation was monitored by the decrease in intensity of the Soret absorption band (λ_{max} = 408 nm), which has been shown to be very sensitive to alteration in the heme environment.[17] $NaClO_4$ and $NaCF_3SO_2O$ were both very effective as denaturants, as may be seen from Fig. 7a; even saturated NaCl solution caused no detectable disruption of the heme from the protein, whereas NaBr showed intermediate efficiency. For the anion denaturation:

$$\text{native-MMb} + n\text{X}^- \rightleftarrows \text{den-MMb}$$

the equilibrium constant

$$K_{DEN} = \frac{[\text{den-MMb}]}{[\text{native-MMb}][\text{X}^-]^n} = \frac{\alpha_{DEN}}{1 - \alpha_{DEN}}[\text{X}^-]^{-n} \quad (3)$$

was determined together with n, the number of molecules of denaturant cooperating in the disruption, for $X^- = ClO_4^-$ (Fig. 7b), assuming that the denaturation is a one-step process as has been found for both urea and guanidine hydrochloride denaturation of equine metmyoglobin.[17]

The acidity of coordinated water in MMb, as determined by spectrophotometric titrations, decreases with increasing ionic strength, as expected for a cationic acid; at an ionic strength of 0.16, the acidity depends on the nature of the anion according to $Cl^- > Br^- > CF_3SO_2O^- > ClO_4^-$ (Table 4). For the sodium chloride and perchlorate media, the dependence of the pK_a value on the salt concentration was analyzed on the basis of the Debye-Hückel theory in the ionic strength region 0.07 to 0.35, and as may be seen from Fig. 8, the effective charge on the Fe(III) center was found to be ca. 3 and ca. 2 for the perchlorate and the chloride media, respectively.

Table 3. pK_a[a] for imidazolium ion and $\Delta H^{\ominus}$ and $\Delta S^{\ominus}$ for acid dissociation of imidazolium ion in aqueous solution of ionic strength 0.16 at 25.0 °C.

Medium	pK_a[a]	$\Delta H^{\ominus}$/kJ mol^{-1}	$\Delta S^{\ominus}$/J mol^{-1} K^{-1}
0.15 M NaCl	7.077(2)	35.6(1.6)	−16(7)
0.16 M $NaClO_4$	7.075(2)	35.9(1.6)	−15(5)
0.16 M NaBr	7.111(2)	39.0(1.6)	− 5(5)
0.16 M $NaCF_3SO_2O$	7.000(2)	37.2(1.7)	− 9(6)

[a] $pK_a = -\log(K_a/\text{mol } l^{-1})$.

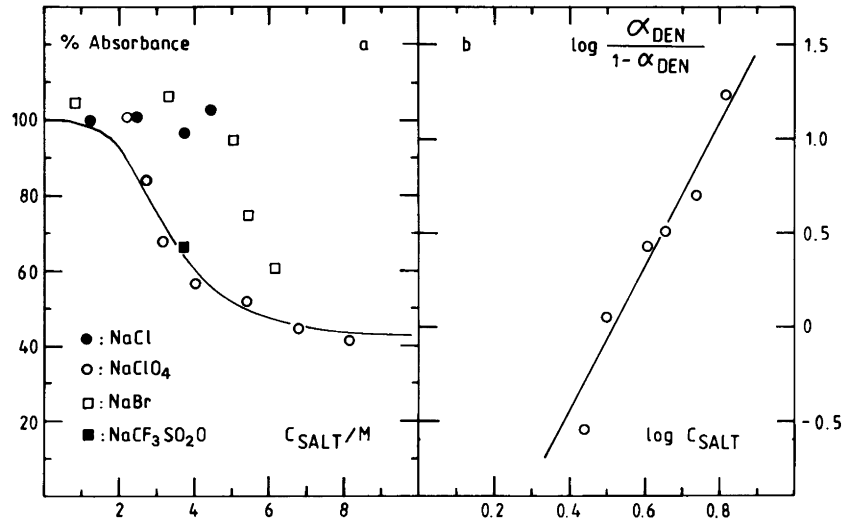

Fig. 7. Denaturation of equine metmyoglobin in aqueous sodium salt solutions with pH close to 7 (5 mM phosphate buffer) at 19 °C as monitored by the decrease of the Soret absorption band (λ_{max} = 408 nm). (a) Absorbance (as percentage of absorbance in buffer without salt) as a function of salt concentration: Sodium chloride has no effect, even in a saturated sodium chloride solution, whereas sodium perchlorate has a transition point (concentration of denaturant required to effect 50 % of the total change) at 3.3 M. Full line calculated for sodium perchlorate as denaturant using the parameters determined in part (b) of this figure. The transition caused by sodium bromide occurs at a higher concentration (above 5 M), although the limited solubility of sodium bromide hampers the exact determination. (b) Determination of K_{DEN}, the equilibrium constant for perchlorate denaturation of metmyoglobin, and *n*, the number of participating perchlorate ions according to eqn. (3): $K_{DEN} = 0.011(7)$ and $n = 3.8(4)$.

Discussion

The rate of acid-catalyzed autoxidation of oxymyoglobin in salt media of physiological ionic strength depends on the nature of the anion rather than on the nature of the cation (Fig. 3 and Table 2), and for the anions included in the present investigation the rate decreases according to a series ($ClO_4^- > CF_3SO_2O^- > Br^- > Cl^-$) which is clearly different from that of decreasing anion nucleophilicity. For both sodium chloride and sodium perchlorate, the kinetic salt effect on the acid-catalyzed autoxidation follows a Debye-Hückel law corresponding to a reaction in which a proton reacts with a monopositive substrate in the chloride medium and with a dipositive substrate in the perchlorate medium, respectively. This difference suggests the binding of a chloride ion near the oxygen-binding Fe(II) in the heme pocket of myoglobin. As may also be seen from Table 5, the same difference in charge, i.e. ca. 1, was found between MMb dissolved in sodium chloride and in sodium perchlorate medium. The effective charge of MMb was determined from the ionic strength dependence on the acidity of the water ligand coordinated to the Fe(III) cen-

Table 4. pK_a[a] for coordinated water in equine metmyoglobin in aqueous salt media at 25.0 °C.

Ionic strength	pK_a^{NaCl}	pK_a^{NaBr}	$pK_a^{NaCF_3SO_2O}$	$pK_a^{NaClO_4}$
0.35	8.81(1)			9.16(1)
0.16	8.75(1)[b]	8.81(3)	8.91(2)	8.99(1)[b]
0.09	8.76(2)			8.90(1)
0.07	8.71(4)			8.90(7)

[a] $pK_a = -\log(K_a/\text{mol } l^{-1})$. [b] At 0.6 °C and ionic strength 0.16: $pK_a^{NaCl} = 9.28(3)$ and $pK_a^{NaClO_4} = 9.57(1)$, giving the following estimates for the acid dissociation: $\Delta H^{\ominus} = 34$ kJ mol^{-1} and $\Delta S^{\ominus} = -54$ J mol^{-1} K^{-1} in 0.16 M NaCl; $\Delta H^{\ominus} = 37$ kJ mol^{-1} and $\Delta S^{\ominus} = -48$ J mol^{-1} K^{-1} in 0.16 M $NaClO_4$.

Table 5. Effective charge in the heme pocket of equine oxymyoglobin and metmyoglobin in aqueous solutions of sodium perchlorate and sodium chloride, as determined from the kinetic salt effect on acid-catalyzed autoxidation of oxymyoglobin and from the ionic strength dependence of acid dissociation of metmyoglobin, respectively.

	Medium		
	$NaClO_4$	NaCl	Δ^c
MbO_2[a]	+2.0±0.1	+1.0±0.1	1
MMb[b]	+2.7±0.2	+1.9±0.3	1

[a]From kinetic salt effect on acid-catalyzed autoxidation in the pH region 5 to 6. [b]From ionic strength dependence of pK_a in the pH region 8 to 10. [c]Difference between perchlorate and chloride media rounded off to nearest integer.

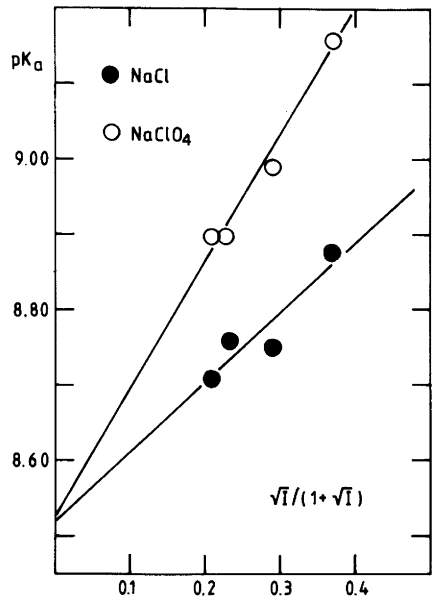

Fig. 8. Water coordinated to Fe(III) in equine metmyoglobin becomes a weaker acid with increasing ionic strength ($0.07 \leq I \leq 0.35$). Values for $K_a = [H^+][MMbOH^{(z-1)+}]/[MMbOH_2^{z+}]$, determined by spectrophotometric titration at 25.0 °C, are plotted according to $pK_a = pK_a^0 + (2z-2)\ 0.51\ \sqrt{I}/(1+\sqrt{I})$. The effect of ionic strength is more significant in sodium perchlorate solution, for which $z = 2.7 \pm 0.2$, than in sodium chloride solution, for which $z = 1.9 \pm 0.3$. pK_a^0 has a common value of 8.52±0.07 in the two salt media.

ter, a method which necessitates a solution pH well above the isoelectric point of the myoglobin. A shift in the total charge of the protein is clearly expected as a result of the change of solution pH and iron oxidation state. It is notable, however, that the difference between the chloride and perchlorate media with regard to the effective charge near the iron center as probed by different chemical methods remains essentially unaltered. The pK_a for MMb in sodium salt solutions of physiological ionic strength depends on the nature of the anions (Table 4: $ClO_4^- > CF_3SO_2O^- > Br^- > Cl^-$) in a fashion that qualitatively parallels that for MbO_2 autoxidation, suggesting that anions are bonded in the heme pocket according to size, thereby reducing the effective charge accordingly.

The same ranking among the anions examined was seen for the salt denaturation of MMb (Fig. 7), and the perchlorate ion is very effective in the disruption of MMb. In contrast, no denaturation was observed for chloride, which may reflect a stabilizing effect of the binding of the latter physiologically important anion to myoglobins.

The globin polypeptide chain, which consists of 153 residues (with 18 substitutions between equine and bovine myoglobin), is attached to the heme moiety through the imidazole ring of the proximal histidine (amino acid number 93),[18] and the heme pocket shields the porphyrin and creates the special environment of the ligand binding site. In oxymyoglobin, dioxygen is bonded as the sixth ligand to Fe(II) and the bound dioxygen is stabilized by hydrogen bonding from an imidazole ring of the distal histidine (number 64), as has been shown by neutron diffraction[19] (see Fig. 9). In solution, a chloride bound in the heme pocket may contribute to this stabilization.

The autoxidation of MbO_2 involves the transfer of one electron from Fe(II) to dioxygen according to eqn. (4), a reaction which is subject

$$Fe(II)O_2^{z+} \rightarrow Fe(III)^{(z+1)+} + O_2^- \qquad (4)$$

both to acid and to base catalysis, and for which several reaction mechanisms have been proposed.[9] The pH of beef ranges from 5.2 to 6.5, and in this pH region the major part of the autoxidation is the result of acid catalysis; the ensuing discussion will therefore focus on this reaction

path. A proton equilibrium prior to rate-determining electron transfer is implicated by the observation of specific rather than general acid catalysis,[20] as has been confirmed for several types of MbO_2 by the absence of any influence on rate of the nature or concentration of the buffer used (cf. Table 1):[10,15,16]

$$Fe(II)O_2^{z+} + HA \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} Fe(II)O_2H^{(z+1)+} + A^- \quad (5)$$

$$Fe(II)O_2H^{(z+1)+} + H_2O \xrightarrow{k_2} Fe(III)^{(z+1)+} + O_2^- + H_3^{O+} \quad (6)$$

For conditions of fixed pH and with excess of the acid HA the rate depends on the hydrogen-ion concentration, and not on the concentration of HA, according to eqn. (7):

$$\frac{d[Fe(III)]}{dt} = \frac{k_2[H^+][Fe(II)]}{[H^+] + (k_{-1}/k_{+1})K_a} \quad (7)$$

where $K_a = [H_3O^+][A^-]/[HA]$, and [Fe(II)] is the concentration of $Fe(II)O_2^{z+}$ plus $Fe(II)O_2H^{(z+1)+}$. The autoxidation rate is proportional to $[H^+]$ for the condition $[H^+] \ll (k_{-1}/k_{+1})K_a$:

$$\frac{d[Fe(III)]}{dt} = k_{H^+}[H^+][Fe(II)] \quad (8)$$

and a kinetic salt effect on the second-order rate constant k_{H^+} is expected [depending on the effective charge of Fe(II)]. In contrast, the first-order rate constant [k_0 of eqn. (2)] for the uncatalyzed autoxidation is expected to be independent of the salt medium. The confirmation of this latter prediction (for chloride and perchlorate, see Figs. 2 and 3) adds support to the anion-binding model discussed above.

A limiting acid-catalyzed autoxidation rate equal to k_2[Fe(II)] will be attained, according to eqn. (7), when $[H^+]$ is increased. Such a levelling in rate, however, has not been observed for the acid-catalyzed autoxidation of MbO_2 for pH > 5, which indicates that the pK_a for the protonization site is well below this limit. The distal histidine

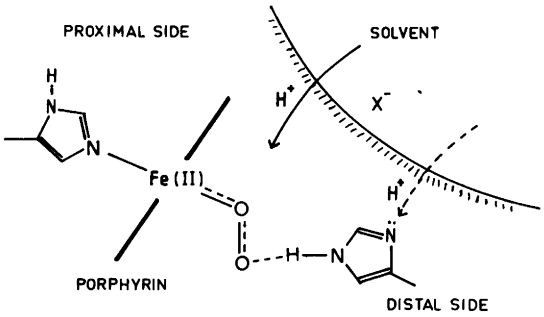

Fig. 9. Dioxygen bonded in MbO_2 is stabilized by hydrogen bonding from the distal histidine,[18] and the imidazole ring of the latter amino acid (pK_a = 6.75) has been proposed to act as a proton-relay in the transformation of the superoxide ion, O_2^-, into the better leaving group HO_2 (broken arrow).[8] Direct protonization of coordinated dioxygen/superoxide (p$K_a \sim -2$) is suggested for more acidic solution or for salt media where the proton-relay is disconnected (arrow).

has been proposed to act as a proton-relay in the acid catalysis, as outlined in Fig. 9, and the deviation from the simple expression of eqn. (2) for k_{obs} in acidic and neutral solution previously noted (and confirmed in the present investigation for both bovine and equine MbO_2 in the chloride medium) has formed the basis for the determination of pK_a = 6.75 at 25 °C for the distal histidine.[7,8]

In contrast to the results for the chloride medium, the simple expression of eqn. (2) is valid for the k_{obs}/pH profile in the perchlorate medium. The proton-relay apparently requires a chloride ion in the heme pocket or, as an alternative explanation, perchlorate induces conformational changes in the heme pocket which disconnect the proton-relay. Given the crucial role of the distal histidine (and the reported notable difference of 0.276(2) in the pK_a of the imidazolium ion in 3 M $NaClO_4$ and 3 M NaCl at 25 °C),[21] a specific imidazole/anion interaction was suspected but could not be confirmed for the 0.16 M salt media in question (Table 3). Other factors, alone or in combination with an imidazole/anion interaction, thus control the anion specificity of the heme pocket, and detergent-induced autoxidation has demonstrated that steric hindrance is one important factor governing the rate of autoxidation.[22]

For reaction conditions where the proton relay is not operative (perchlorate medium or chloride medium below pH = 5.5), the pK_a of the protonization site is well below 5 and is closely related to the nature of the iron center (the charge transfer – Fe(II),O_2 → Fe(III),O_2^- – is expected to be facilitated by the protonization of the superoxide ion). Coordinated H_2O in MMb has a $pK_a \sim 9$ to be compared with $pK_a \sim 16$ for water, and HO_2 coordinated to Fe(III) is accordingly expected to have $pK_a \sim -2$, assuming a pK_a value of 4.8 for HO_2.[23] Direct protonization of coordinated O_2^-, generating the better leaving ligand HO_2, is thus in agreement with the observed specific acid catalysis without a limiting rate for pH > 5.

In conclusion, the kinetic salt effect on the acid-catalyzed autoxidation of oxymyoglobin in different salt media differs as a result of differences in anion binding to the protein. Chloride reduces the effective positive charge near the iron centre in the heme pocket by one unit compared to the non-binding perchlorate, and under physiological conditions this chloride ion binding has been found to be important for the conformational stabilization of the heme pocket, especially with respect to the role of the distal histidine. Moreover, chloride modifies the chemical properties of oxymyoglobin in a manner similar to that demonstrated for the cytochromes,[24] and for sodium chloride the increase in autoxidation rate caused by an increased ionic strength is levelled at a value expected for a protein with an effective positive charge of one. However, when sodium chloride in conventional amounts is added to ground beef or other meat products the ionic strength increases from approximately 0.16 to 0.35, resulting in a 30% rate increase for the autoxidation, a rate enhancement comparable to that caused by the postmortem pH decrease.

Acknowledgement. This research was supported by a joint grant from the Danish Technical Research Council and the Danish Agricultural and Veterinary Research Council. Grethe Lis Hansen and Anna Marie Buch are thanked for technical assistance.

References

1. George, P. and Stratmann, C.J. *Biochem. J. 57* (1954) 568.
2. Gotoh, T. and Shikama, K. *J. Biochem. 80* (1976) 397.
3. Wallace, W.J., Houtchens, R.A., Maxwell, J.C. and Caughey, W.S. *J. Biol. Chem. 257* (1982) 4966.
4. Watts, B.M. *Adv. Food Res. 5* (1954) 1.
5. Bertelsen, G. and Skibsted, L.H. *Meat Sci. 19* (1987) 243.
6. Andersen, H.J., Bertelsen, G. and Skibsted, L.H. *To be published.*
7. Shikama, K. and Sugawara, Y. *Eur. J. Biochem. 91* (1978) 407.
8. Sugawara, Y. and Shikama, K. *Eur. J. Biochem. 110* (1980) 241.
9. Shikama, K. *Biochem. J. 223* (1984) 279.
10. Gotoh, T. and Shikama, K. *Arch. Biochem. Biophys. 163* (1974) 476.
11. Skibsted, L.H. and Ford, P.C. *Acta Chem. Scand., Ser. A 34* (1980) 109.
12. George, P. and Hanania, G. *Biochem. J. 52* (1952) 517.
13. Skibsted, L.H. *Acta Chem. Scand., Ser. A 33* (1979) 113.
14. Bowen, W.J. *J. Biol. Chem. 179* (1948) 235.
15. Suzuki, T. and Shikama, K. *Arch. Biochem. Biophys. 224* (1983) 695.
16. Suzuki, T., Sugawara, Y., Satoh, Y. and Shikama, K. *J. Chromatogr. 195* (1980) 277.
17. Schechter, A.N. and Epstein, C.J. *J. Mol. Biol. 35* (1968) 567.
18. Dayhoff, M.O. *Atlas of Protein Sequence and Structure,* National Biomedical Research Foundation, Washington, D.C. 1976, Vol. 5, Suppl. 2.
19. Phillips, S.E.V. and Schoenborn, B.P. *Nature (London) 292* (1981) 81.
20. Laidler, K.J. *Chemical Kinetics,* 3rd ed., Harper and Row, New York 1987.
21. Sjöberg, S. *Acta Chem. Scand. 26* (1972) 3400.
22. Kasuga, K., Nakao, S., Kamezawa, T., Tsukahara, K. and Yamamoto, Y. *Biochem. Biophys. Acta 788* (1984) 313.
23. Fridovich, I. *Ann. Rev. Biochem. 44* (1975) 147.
24. Margalit, R. and Schejter, A. *Eur. J. Biochem. 32* (1973) 492.

Received November 25, 1987.