The Breakdown of 2,3-Bis(phospho)-D-glycerate by Fe(III)-Haemoglobin

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Fe(III)-haemoglobin is shown to catalyse the hydrolysis of 2,3-bis(phospho)-D-glycerate to P_i and 3-phosphoglycerate, although the rate is slow, even when the protein is present at concentrations in the millimolar range. The rate of hydrolysis is proportional to the substrate concentration up to at least 10 mM, so if the process is enzymic, the K_m must be high.

Haemoglobin has long been regarded as the archetype of allosteric enzymes, although, to date, only binding functions have been attributed to it. In this paper we present evidence to show that Fe(III)-haemoglobin catalyses the hydrolysis of the allosteric effector 2,3-bis(phospho)-D-glycerate.

Experimental

Haemolysates were prepared from human blood and freed from organic phosphates by chromatography on Sephadex G-25 in 0.1 M-NaCl (Benesch *et al.*, 1968). The main haemoglobin fraction A_{II} was isolated by chromatography on DEAE-Sephadex (Atha & Ackers, 1974). The haemoglobin content of the haemolysates or of the purified haemoglobin was increased up to as much as 20 g/100 ml with an Amicon CEC 1 Thin Channel Concentrator (Amicon Corp., Lexington, MA, U.S.A.). Oxy- and deoxy-haemoglobin solutions were prepared by equilibration with pure O_2 and pure N_2 respectively in IL 237 tonometers (Instrumentation Laboratory Inc., Lexington, MA, U.S.A.). Fe(III)-haemoglobin was prepared by the complete oxidation of haemoglobin with $NaNO_2$ (Antonini & Brunori, 1971). The concentrations of haemoglobin and Fe(III)-haemoglobin were routinely determined with a Micro Blood Analyser (Carlo Erba, Milan, Italy), which had been found to agree with chemical methods of determination (van Kampen & Zijlstra, 1965; Evelyn & Malloy, 1938) to within 1%. Isoelectric focusing of haemoglobin was carried out on LKB Ampholine polyacrylamide-gel plates, pH range 3.5–9.5 (LKB, Stockholm, Sweden). 2,3-Bis(phospho)-D-glycerate was determined spectrophotometrically by the procedure of Lowry *et al.* (1964) by using reagents supplied by Sigma (London) Chemical Co. (Poole, Dorset, U.K.). Estimations of P_i was performed by the method of Taussky & Shorr (1953). The reaction between Fe(III)-haemoglobin and 2,3-bis(phospho)-D-glycerate was studied at 37°C in solutions containing 0.1 M-NaCl and 0.1 M-sodium

phosphate, pH 7.0. The phosphate buffer was replaced by 0.2 M-imidazole/HCl, pH 7.0, for those experiments in which the release of P_i was followed. The rates of breakdown of 2,3-bis(phospho)-D-glycerate and of production of phosphate were determined from duplicate concentration measurements at $\frac{1}{2}$ h intervals for 2½ h and by fitting the best straight line through the points. The separation of 2-phosphoglycerate, 3-phosphoglycerate and 2,3-bis(phospho)-D-glycerate by paper chromatography was performed by the procedure of Bandurski & Axelrod (1951).

Results and Discussion

In an investigation of the factors leading to loss of added 2,3-bis(phospho)-D-glycerate from stripped haemolysates, we found that contaminating enzymes present in the haemolysates did not appear to be responsible, since 2,3-bis(phospho)-D-glycerate was stable in solutions of deoxy- and oxy-haemoglobin prepared carefully to avoid the production of Fe(III)-haemoglobin (Fig. 1).

Experiments in which 2,3-bis(phospho)-D-glycerate had been observed to break down showed an apparent correlation with the proportion of the haemoglobin in the oxidized [Fe(III)] form. We therefore carried out a more systematic study of the dependence of the rate of loss of 2,3-bis(phospho)-D-glycerate on its concentration and on the concentration of chemically prepared Fe(III)-haemoglobin. The experiments were performed on haemolysates, but measurements on the rate of breakdown of 2,3-bis(phospho)-D-glycerate by Fe(III)-haemoglobin prepared from a purified sample of haemoglobin showed no difference compared with the rates in haemolysates (these experiments are shown plotted with the others in Fig. 3).

At a given concentration of Fe(III)-haemoglobin, rates of reaction were proportional to the concentration of 2,3-bis(phospho)-D-glycerate. Because of the high protein concentration needed (7–20 g/100 ml) the volume of water available for solution of 2,3-bis-

Fig. 1. Stability of 2,3-bis(phospho)-D-glycerate in solutions of oxy- and deoxy-haemoglobin

■, 0.1M-Phosphate buffer at pH 7.0; ●, oxyhaemoglobin solution; ○, deoxyhaemoglobin solution; Curve (A) for a solution of 1.8mM-haemoglobin with 2% present as Fe(III)-haemoglobin in 0.1M-phosphate buffer, pH 7.0 at 37°C; curve (B) for a solution of 1.8mM-haemoglobin with 9.5% present as Fe(III)-haemoglobin in the same conditions as above; curve (C) calculated for 1.8mM-haemoglobin entirely in the Fe(III)-form by using the relationship shown in Fig. 3.

Fig. 2. Rate of breakdown of 2,3-bis(phospho)-D-glycerate divided by the concentration of 2,3-bis(phospho)-D-glycerate plotted against Fe(III)-haemoglobin concentration

Concentrations of 2,3-bis(phospho)-D-glycerate were in the range 2–9.5mM, and the error bars show the s.d. of the mean result at each concentration of Fe(III)-haemoglobin. The best-fit line has a slope of 0.019 ± 0.002 and intercept -0.0054 ± 0.0019 . The intercept is not significantly different from zero ($0.1 < P < 0.05$).

(phospho)-D-glycerate is substantially less than the volume of the solution. Correction for this was made, assuming that the partial specific volume of haemoglobin is 0.75 ml/g, and that the protein binds 0.20 ml

Fig. 3. Dependence of the rate of breakdown of 2,3-bis(phospho)-D-glycerate on its concentration

○, Results obtained by measurements of 2,3-bis(phospho)-D-glycerate concentrations with the concentration of Fe(III)-haemoglobin from haemolysates in the range 1–3.5mM, in 0.1M-sodium phosphate (pH 7.0)/0.1M-NaCl at 37°C; □, results obtained by measurements of 2,3-bis(phospho)-D-glycerate concentrations with the concentration of Fe(III)-haemoglobin from purified haemoglobin at 1.35mM and in conditions as above; ●, results obtained by measurement of the concentration of P_i with 2.15mM-Fe(III)-haemoglobin in 0.2M-imidazole/HCl (pH 7.0)/0.1M-NaCl, at 37°C.

Fig. 4. Time course of breakdown of 2,3-bis(phospho)-D-glycerate by 2.85mM-Fe(III)-haemoglobin

(A) Best-fit line used for calculating the rate of hydrolysis; (B) second-order progress curve calculated to give the same initial rate; (C) as (B) except that each of the four subunits of Fe(III)-haemoglobin is assumed to be a reactant.

of water/g (Tanford, 1961). The best-fit values of rate per unit concentration of 2,3-bis(phospho)-D-glycerate were plotted against the concentration of Fe(III)-haemoglobin (Fig. 2). The rate of hydrolysis is

shown to be proportional to Fe(III)-haemoglobin concentration, and so in presenting the dependence of the rate on concentration of 2,3-bis(phospho)-D-glycerate (Fig. 3) we have plotted rates per unit concentration of Fe(III)-haemoglobin.

Although the rate of breakdown of 2,3-bis(phospho)-D-glycerate is proportional to the concentration of both 2,3-bis(phospho)-D-glycerate and Fe(III)-haemoglobin, we believe that the latter is acting catalytically and not as a second reactant for the following reasons.

(i) If the Fe(III)-haemoglobin were a reactant, then the plots of 2,3-bis(phospho)-D-glycerate concentration against time would have shown marked curvature over the period of observation in experiments such as the one illustrated in Fig. 4. This would be the case whether one site or four sites per molecule of Fe(III)-haemoglobin reacted.

(ii) Examination of the Fe(III)-haemoglobin by isoelectric focusing after prolonged incubation (up to 12h) with 2,3-bis(phospho)-D-glycerate showed no detectable change in the electrophoretic pattern, which implies that it is indeed acting catalytically and remains unchanged, since the reactants and products are charged and would shift the isoelectric point if incorporated into the molecule.

(iii) The fate of the 2,3-bis(phospho)-D-glycerate was established. One product was shown to be P_i , which was formed in the ratio of 1.00 ± 0.06 (11 determinations) mol/mol of 2,3-bis(phospho)-D-glycerate. The dephosphorylated product was shown to be 3-phosphoglycerate by paper chromatography of the reaction products.

Conclusion

Within the erythrocyte, the steady-state concentration of 2,3-bis(phospho)-D-glycerate is about 4.5 mM,

and its breakdown is catalysed by a phosphatase with a maximum rate of 0.35 mmol/h per l of cells and a K_m of 17 μ M (Rapoport *et al.*, 1974). Fe(III)-haemoglobin, for which the K_m is well above the range of concentrations of 2,3-bis(phospho)-D-glycerate used here (i.e. in excess of 13 mM), is certainly not this phosphatase. It remains to be seen whether Fe(III)-haemoglobin contributes significantly to 2,3-bis(phospho)-D-glycerate breakdown in physiological conditions. Only if all the haemoglobin in the erythrocytes were in the Fe(III) form could it account, in our experimental conditions, for the observed extent of 2,3-bis(phospho)-D-glycerate breakdown.

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