

Kinetics of the Reaction of Hemoglobin with Ethylisocyanide

INTERPRETATION OF THE RESULTS WITHIN A DIMER SCHEME

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SUMMARY

The kinetics of the reaction of human deoxyhemoglobin with ethylisocyanide has been studied, by rapid mixing, over a 50- to 100-fold range of ligand concentration, both as a function of protein concentration (from 3 to 30×10^{-6} M) and ionic strength (from 0.2 to 2.2 M). The results show that the progress curve, which is autocatalytic at high ligand concentration, tends to change shape as the ethylisocyanide concentration is decreased, and finally becomes markedly diphasic.

The experimental results can be fitted satisfactorily with a simple dimer scheme, with only two combination and two dissociation velocity constants. Consideration of these results, in conjunction with other data, allows us to arrive at important conclusions concerning the kinetic origin of cooperativity as observed at equilibrium. The most significant of these is that, to a major degree, cooperative ligand binding finds its kinetic justification in a large decrease of the dissociation velocity constant as the reaction proceeds.

Equilibrium data on the reaction of hemoglobin with ligands unequivocally show that there must be effects beyond those operative in the dimer, since the Hill parameter (n) is higher than two (notably 2.2 to 2.4 for ethylisocyanide (13, 14). However, to the extent to which dimer-dimer interactions are small in comparison with intradimer effects, a dimer scheme, although admittedly an oversimplification, should be able to accommodate the main trends of kinetic behavior of the system.

Even with the limitations stated above, a dimer scheme appears adequate to give a satisfactory fitting of the experimental data reported here, a fact which allows us to arrive at important conclusions concerning the kinetic origin of the cooperative effects observed in equilibrium experiments. The most significant of these is that, to a major degree, functional homotropic interactions manifest themselves in the dissociation velocity constants. It should be emphasized that this conclusion, which sets a new point of view in the kinetic interpretation of cooperative phenomena in hemoglobin, is to some extent independent from the adoption of a dimer model.

EXPERIMENTAL PROCEDURE

Human hemoglobin was prepared with the ammonium sulphate procedure (15) and was stored as the deoxy derivative in a tonometer. Handling of materials was always strictly anaerobic, however a small amount of sodium dithionite (~1 mg per ml) was added in all cases to ensure complete absence of oxygen. Ethylisocyanide was prepared according to the method of Jackson and Kusick (16). Its concentration was checked by stoichiometric titrations with the isolated α chains (14).

Kinetic measurements were performed with a Gibson-Durrum stopped-flow apparatus equipped with a 2-cm observation tube. The measured dead time is 3.5 msec.

Computations were made on a Solartron (England) HS-7/1 analogue computer.

RESULTS

The kinetics of combination of deoxyhemoglobin with ethylisocyanide has been studied over a 50- to 100-fold range of ligand concentration, both as a function of protein concentration (from 3 to 30×10^{-6} M in heme) and ionic strength (from 0.2 to 2.2 M). The effect of pH from 6 to 9.1 has been investigated also, but will only be briefly mentioned here.

The results of an experiment on the combination of deoxyhemoglobin with ethylisocyanide are reported in Fig. 1 in terms

Recent work has shown that, in spite of large differences in the absolute values of the rates, the kinetics of the reactions of hemoglobin with different ligands is similar. Thus, under conditions where the "on" rates predominate, *i.e.* at very high ligand concentration, the time course of the combination shows an acceleration as saturation with the ligand increases. At low ligand concentration, when the "off" rates should contribute substantially to the attainment of equilibrium, the shape of the approach to equilibrium changes character (1-5).

The present paper contains the results of an extensive investigation of the reaction of human hemoglobin with ethylisocyanide and an attempt to interpret them in terms of a simplified dimer scheme. This approach presupposes that even in tetrameric hemoglobin ($\alpha\beta$) dimers are the fundamental units of function, an idea which appears justified by the equilibrium and kinetic behavior of hemoglobin under conditions favoring dissociation into dimers (6-8)¹ and of artificial intermediates in which one type of chain is frozen in the ligand-bound conformation (2, 9-12).

¹ G. Amiconi, E. Antonini, M. Brunori, E. Chiancone, and P. Vecchini, manuscript submitted for publication.

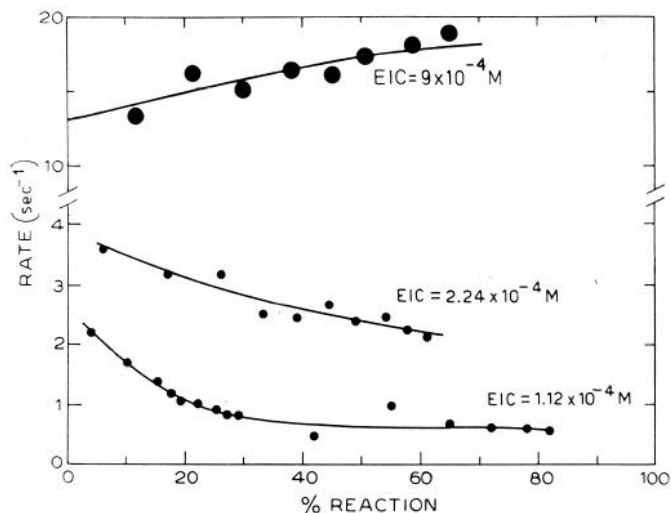


FIG. 1. Combination of deoxyhemoglobin with ethylisocyanide (EIC). Apparent rate constant as a function of percentage reaction at different ligand concentrations (indicated). Conditions: 0.2 M potassium phosphate buffer, pH 7, 18°. Hemoglobin concentration: 3.4×10^{-5} M (in heme). Observations at 532 nm.

of apparent rate constant, calculated over a small time interval, as a function of the percentage reaction. At constant protein concentration, the shape of the progress curve changes markedly as the ligand concentration is decreased. Thus at high ethylisocyanide concentration there is a significant increase in the apparent rate constant as hemoglobin becomes saturated with the ligand, and in this respect the reaction with ethylisocyanide resembles that with other ligands, notably O_2 or CO (3, 5, 17). As the ligand concentration is decreased, the autocatalytic shape of the progress curve tends to vanish, and finally, at low concentrations, the shape becomes markedly diphasic, with a decrease in the apparent rate constant as the reaction proceeds. Changing the protein concentration from 30 to 3×10^{-6} M and the buffer from 0.1 M phosphate to 0.1 M phosphate + 2 M NaCl did not alter significantly the behavior of the system. At pH 9.1 in borate buffer the kinetics was qualitatively similar to that at pH 7; however the transition from the autocatalytic to the diphasic time course occurred at lower ligand concentrations.

DISCUSSION

Hemoglobin kinetics has been described in the past by a four-stage Adair scheme, with four "on" and four "off" constants (2, 5); this scheme is obviously bound to give a fit of the data better than that obtainable with a dimeric scheme, and particularly should be able to account for the equilibrium behavior ($n > 2$). However, it should be realized that a four-stage Adair model is, in itself, an oversimplification and its advantages as a formal model are overcome by the possibility of obtaining degenerate solutions as a result of the fairly large number of disposable constants.

In view of this, as far as only an approximate solution can be obtained at present, it may be convenient to search for the simplest model capable of accommodating the most relevant features of the system. Thus, in so far as even in tetrameric hemoglobin ligand binding is dominated by the behavior of the dimer, the use of a two-stage model appears to be justified as a plausible approach to a quantitative description of the system.

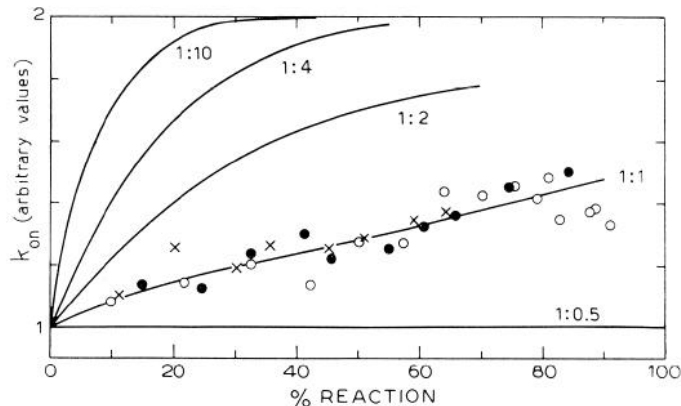
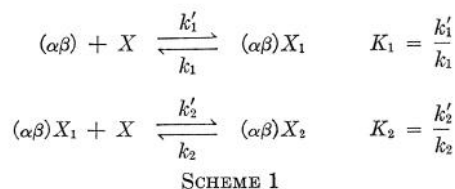


FIG. 2. Combination of deoxyhemoglobin with ethylisocyanide, under the following conditions. ○, Hemoglobin concentration = 3×10^{-6} M; ethylisocyanide concentration = 6.2×10^{-4} M; 0.2 M potassium phosphate, pH 7, wave length = 428 nm. ●, Hemoglobin concentration = 3×10^{-6} M; ethylisocyanide concentration = 6.2×10^{-4} M; 0.2 M potassium phosphate, pH 7 + 2 M NaCl; wave length = 428 nm. ×, Hemoglobin concentration = 3.4×10^{-5} M; ethylisocyanide concentration = 9×10^{-4} M; 0.2 M potassium phosphate, pH 7; wave length = 532 nm.

In its simplest form a dimer scheme corresponds to the following:



At high enough ligand concentration, the kinetics of ligand binding is essentially dominated by k'_1 and k'_2 ; their ratio determines the shape of the progress curve, which will be independent of the ligand concentration and of the absolute rate of the process. A series of computed curves, showing values of the apparent second order rate constant versus progress of reaction for different ratios of $k'_1:k'_2$, is shown in Fig. 2. Comparison of the experimental data with these curves yields immediately estimates of the relative value of k'_1 and k'_2 for the reaction with ethylisocyanide.

The value of k_2 , the dissociation velocity constant corresponding to the second step in Scheme 1, may be estimated from displacement experiments in which ethylisocyanide-hemoglobin is mixed with CO, according to the treatment of Gibson and Roughton (see Reference 5) adapted to Scheme 1. The time course of the displacement of ethylisocyanide by CO shows a certain amount of heterogeneity, and yields a value of k_2 of 0.1 to 0.4 sec^{-1} .

The approximate values of k'_1 , k'_2 , and k_2 , obtained from experiments, are set into an analogue computer program made according to Scheme 1, and the experimental data are fitted with only one freely disposable constant (namely k_1), although a certain variability in the values of the other three constants is also allowed.² A satisfactory fit of the experimental results is obtained, over the whole concentration range, with the values of the rate constants reported in Table I (Fig. 3). The shape of the experimental curves deviates from the calculated ones in

² The variability corresponds to approximately $\pm 30\%$ for k'_1 and k'_2 , while k_2 was changed between 0.1 and 0.4 sec^{-1} .

TABLE I

Kinetic constants for reaction of human hemoglobin with ethylisocyanide

Constants are defined in Scheme 1.

k'_1	k_1	k'_2	k_2
$M^{-1} sec^{-1}$	sec^{-1}	$M^{-1} sec^{-1}$	sec^{-1}
2.7×10^4	8	2.7×10^4	0.16

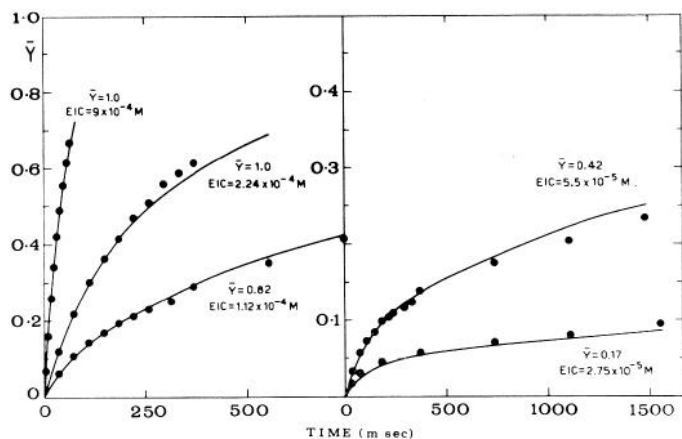


FIG. 3. Combination of deoxyhemoglobin with ethylisocyanide (EIC) at various ligand concentrations (indicated). Conditions: Hemoglobin concentration = 3.4×10^{-5} M; 0.2 M potassium phosphate, pH 7; 18°; wave length = 532 nm. Continuous lines are analogue computer solutions according to Scheme 1 with the constants reported in Table I (see text).

the higher saturation range, a fact which may be taken as an indication of the limits of the model, obviously oversimplified and inadequate to give values of n higher than two. In spite of this, the reproduction, with a minimum number of constants, of the main features of the system gives confidence that Scheme 1 represents a good approximation to the real situation.

It should be made clear that adoption of such a scheme relies on two basic assumptions. (a) Intramolecular conformational transitions do not appear as separate reaction steps. (b) the α and β chains are exactly equivalent in the reaction with the ligand. Moreover protein-dependent effects arising from ligand-linked dissociation processes have not been taken into account. These, however, are negligible under the conditions in which most of the experiments reported here have been made.

Assumption *a* appears to be justified since there is strong evidence that conformational transitions in hemoglobin are very fast and therefore never become rate limiting at the ligand concentrations normally achieved (3, 4, 18, 19).

Assumption *b*, however, appears to be valid only to a first approximation. The α and β chains may be equivalent, or nearly so, in the reaction of hemoglobin with O_2 and CO but not in the case of other ligands. Clear indication of intramolecular heterogeneity has been obtained in the kinetics of oxidation of hemoglobin by ferricyanide (20) and in the reaction of ferrihemoglobin with azide (21). In the case of ethylisocyanide nonequivalence between α and β chains is suggested by the following evidence. (a) The time course of the replacement of ethylisocyanide by CO in hemoglobin does not correspond to a homogeneous reaction, since the rate tends to decrease as the reaction proceeds. (b) The low value of n in equilibrium experi-

ments (14) may reflect slightly different "intrinsic" affinities of the two types of chains. (c) The isolated α and β chains are not identical in the reaction with this ligand: k_{on} and k_{off} for β chains are about 2 times larger than for α chains.³ However nonequivalence of the two types of chains in the reaction of hemoglobin with ethylisocyanide is not of such a degree as to affect the main conclusions reached here about the size of the four kinetic constants involved in Scheme 1.

If a simple dimer scheme is accepted as a plausible description of the real situation, the following conclusions concerning hemoglobin kinetics can be made.

1. The change in shape of the progress curve with ligand concentration arises, in the low concentration range, from contributions due to the dissociation velocity constants. Because of this, the differences in the shape of the progress curves between different ligands (reported up to now) are caused by different ratios of the "on" and "off" velocity constants. When this is taken into account all the ligands behave similarly.

2. The autocatalytic shape of the progress curve observed at high ligand concentration is explained, within a dimer scheme, by values of the combination velocity constant for the second step only slightly higher than their statistical values. The ratio $k'_2:k'_1$ may be different for the various ligands varying from 4 to 1. This implies that in rapid mixing experiments starting with deoxyhemoglobin at no stage during the course of reaction the hemes acquire a reactivity anywhere near that of the isolated chains, or of the quickly reacting forms observed in flash photolysis experiments. This conclusion is in agreement with the kinetic behavior of artificial intermediates, for which the combination velocity constant with CO is only slightly higher than the over-all value observed with hemoglobin (9, 12).

3. The diphasic approach to equilibrium, observed at low ligand concentration, is caused by a large difference between the dissociation velocity constants for the first and second reaction step in Scheme 1. In this perspective the substantial cooperativity observed in the dimer finds its kinetic counterpart into a large decrease of the dissociation velocity constants as the reaction proceeds, and only to small effects on the combination velocity constants. In other words, it is the rapidity of dissociation of the first ligand molecule attached to deoxyhemoglobin which accounts, primarily, for the lower value of the first equilibrium constant.

The picture outlined above may, to a first approximation, account for the kinetic behavior of hemoglobin observed in relaxation experiments, where, at high protein concentration, two relaxation processes have been found (4). The difference between the two relaxation times, which corresponds to a factor of 10 to 20 may be related to the large difference between the dissociation velocity constants of the first and the second step in Scheme 1.

As far as the flash photolysis experiments are concerned, their interpretation is, at least in part, still obscure (2, 5). The greater difference between them and the rapid mixing experiments is that the reaction is initiated by sudden removal of the ligand from ligand-bound hemoglobin. Therefore, the kinetics by flash photolysis may reflect the special reactivity of molecular species present in ligand-bound hemoglobin solutions, which do not appear significantly in other kinetic experiments.

³ B. Talbot, M. Brunori, and E. Antonini, unpublished results.

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