Properties of Trout Hemoglobins Reconstituted with Unnatural Hemes†

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ABSTRACT: Native globins isolated from trout hemoglobin components I and IV have been reconstituted with proto-, meso-, and deuteroheme, and the spectral and functional properties of the reconstituted hemoglobins have been investigated. Equilibrium and kinetic studies allow the following conclusions. (a) The properties of the proto-reconstituted hemoglobins are very similar, or indistinguishable, from those of the native Hb's I and IV. (b) The CO binding kinetics for both proteins were found to be consistent with the equilibrium data: the overall association rate constant increases (and the autocatalytic character of the reaction decreases) in the order proto, meso, deutero. (c) A marked pH dependence of both ligand affinity and cooperativity is maintained in the reconstituted Hb's IV: at pH 6 the fractional saturation with oxygen in air (Root effect) is lower for proto- than for meso- and deutero-Hb IV. The results obtained, including partial photodissociation experiments at different pH values, can be considered, to a first approximation, consistent with the basic features of a simple two-states model.

The role of heme–globin interactions in regulating the functional properties of hemoglobin has been emphasized in the stereochemical model proposed on the basis of crystallographic studies (Perutz, 1970). Perturbation of these interactions may come about on reconstitution of native apohemoglobin with different iron porphyrins, as shown very clearly by a number of studies on the structural and functional properties of human hemoglobin (Antonini et al., 1964; Antonini & Brunori, 1971). The changes in the ligand-binding properties of hemoglobin reconstituted with modified hemes have been attributed to alterations in the electronic structure of the iron porphyrin and (or) to differences in the steric effects of the 2,4 substituents (Seybert et al., 1976; Asakura & Sono, 1974). Studies of reconstituted hemoglobins from pigments of other species, besides leading to a deeper knowledge of specific structure–function relationships, may be relevant to the problem outlined above.

In this perspective, and with the idea of getting a better insight in the allosteric properties of fish hemoglobins characterized by a Root effect (Brunori, 1975), we studied the functional properties of the major hemoglobin components from trout (Hb trout IV and I) reconstituted with proto-, meso-, and deutero-heme. This investigation was made possible by the development of a suitably modified acid–butanone method which allowed us to obtain, for the first time, native globin(s) from fish hemoglobin(s) (Fioretti et al., 1976).

Experimental Procedures

Hemoglobin components I and IV were prepared from trout's blood as previously described (Binotti et al., 1971).

Globins were isolated at pH 2.8 from ferric Hb I and Hb IV, using a modified butanone-extraction procedure (Fioretti et al., 1976; Teale, 1959; Yonetani, 1967).

Reconstituted hemoglobins with proto-, meso-, or deuteroheme were obtained as follows. A heme solution in 1 mM NaOH (minimum volume) was slowly added in stoichiometric amounts to a gently stirred aqueous solution of freshly prepared 0.3–0.5 mM globin (I or IV). Then 1 M phosphate buffer (pH 6.0) was added to reach the final concentration of 0.01 M (pH 6.0). The solution was then clarified by centrifugation and passed through a Sephadex G-25 column equilibrated with 0.01 M phosphate buffer (pH 6.0). The eluted brown solution, freed from excess heme, was than applied on a carboxymethylcellulose (CM 50) column in the same buffer and eluted with 0.1 M phosphate buffer (pH 7.0). Under these conditions, the first fraction eluted from the column represented the reconstituted native protein. This purified material was reduced by sodium dithionite and either used directly for the equilibrium and kinetic experiments with CO, or passed through a Sephadex G-25 column to obtain the oxygenated derivative for the oxygen binding studies. All operations were performed at 4 °C.

Hemes. Crystalline hemin was purchased from Sigma and used directly. Meso- and deuterohemes were prepared as previously published (Smith, 1975). Their concentration was checked spectrophotometrically using the molar extinction coefficients of the corresponding reduced pyridine hemochromogen (Antonini & Brunori, 1971).

Heme Titrations. Titration of globin I and IV with the various hemes was performed at 10 °C measuring the increase in the optical density at 400 nm observed upon addition of increasing amounts of the heme to a solution of globin $\sim 5 \times 10^{-6}$ M. Each addition corresponded to an increase of heme concentration of about 1 μM. Equilibrium was reached instantaneously on the spectrophotometric time scale.

Heme content of the reconstituted proteins was determined by the reduced pyridine hemochromogen method (Antonini & Brunori, 1971).

Oxygen and carbon monoxide equilibria were followed spectrophotometrically in the Soret region, using previously published procedures (Rossi Fanelli & Antonini, 1958; Kertesz et al., 1965).

Flash photolysis experiments were performed using the apparatus previously described (Antonini et al., 1967).

Dry weight determinations on globins were obtained with a Cahn microbalance, after heating at 104 °C to constant weight.

Circular dichroism (CD) spectra of globin in water have

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TABLE I: Spectral Data of Reconstituted Hb I and IV (Phosphate Buffer (0.2 M), pH 7.8).

	Oxy		Deoxy		Carbonmonoxy	
	λ_{max}	ϵ (mM)	λ_{max}	ϵ (mM)	λ_{max}	ϵ (mM)
Native Hb IV	547	14.4	555	12.3	566	12.9
	539	13.6	430	118.6	536	13.6
	412				418	187.5
Reconstituted proto-Hb IV	574	12.8	555	12.6	566	13.1
	538	12.3	429	116.5	536	13.3
	412	111.3			418	170.0
Reconstituted meso-Hb IV	564	11.3	545	12.6	555	11.5
	531	12.7	419	112	527	12.8
	402	113.2			408	187.0
Reconstituted deutero-Hb IV	562	9.4	542	10.3	553	9.5
	529	12.0	418	112.4	526	12.2
	400	120.4			407	201.5
Native Hb I	574	14	554	12.2	566	12.8
	539	13.1	430	122.0	536	13.1
	413	123.0			418	183.0
Reconstituted proto-Hb I	574	13.0	554	12.5	566	12.8
	539	12.4	430	113.0	536	13.3
	411	115.0			418	165.2
Reconstituted meso-Hb I	565	11.8	545	12.0	555	12.0
	531	12.5	421	114.0	527	13.1
	402	125.0			407	204.0
Reconstituted deutero-Hb I	562	10.8	544	11.0	555	7.9
	531	13.2	419	113.0	527	10.2
	401	118.0			408	170.0

been recorded in a Cary 60 spectropolarimeter with a 6002 CD attachment, in 0.1-cm cells at 10 °C. Ellipticity values were calculated on a mean residue weight basis (mol wt = 112).

Absorption spectra were recorded in a Cary 118 or in Beckmann DBGT spectrophotometers.

Results and Discussion

Preparation and Some General Properties of Globins. As previously reported (Fioretti et al., 1976) the butanone method gave satisfactory results and achieved complete heme extraction at pH 2.8.

After purification on a Sephadex G-75 column equilibrated with water, two fractions were collected, the second one containing about 80% of the total protein and being ~80% titratable with protoheme. This fraction can be stored in the cold only for a few days, since it becomes turbid and loses (specific) heme binding capacity under a variety of experimental conditions (different buffers, ionic strengths, and pH values).

The extinction coefficients, $E_{280}{}^{\%}$, obtained from dry weight and optical absorption determinations, are: 12 ± 1 and 11 ± 1 (Fioretti et al., 1976) for globin I and globin IV, respectively. These absorption values are higher than that reported for human apohemoglobin ($E_{280}{}^{\%}$ = 8.0). This is in agreement with the higher tryptophan content found in these hemoglobins, since from their amino acid sequences, which are ~90% solved, 10 and 8 tryptophan residues per tetramer have been found in Hb I and IV, respectively (Bossa et al., 1976, and personal communication). The circular dichroism spectra of both globins between 200 and 250 nm show the presence of two negative peaks at 208 and 222 nm typical of structures containing a substantial fraction of the residues in an α-helical conformation. The molar ellipticities values at 222 nm are −15 000 and −14 000 deg cm^2/dmol for globin I and IV, respectively,

TABLE II: Oxygen Equilibrium Data of Reconstituted Trout Hemoglobins I and IV (0.05 M Phosphate, 20 °C).

	pH	Log $p_{1/2}$	$n_{1/2}$
Hb IV (native)	7.40	1.5	2.2
	7.70	1.28	2.1
Proto-Hb IV	7.40	1.35	2.1
	7.80	1.15	1.85
Meso-Hb IV	7.25	0.85	1.6
	7.80	0.69	1.6
Hb I (native)	7.60	1.15	2.6
Proto-Hb I	7.40	0.99	2.3
Meso-Hb I	7.40	0.22	2.0

and would correspond to an α-helical content of about 40% (Greenfield & Fasman, 1969), which is comparable to, but a little lower, than that found for other globins (Beychok, 1967). These results confirm previous observations of a reduction of the α-helical content of the heme proteins upon removal of the heme moiety (Antonini & Brunori, 1971), if one recalls that the α-helical contents of Hb I and Hb IV, as calculated from their $[\theta]_{222}$ (Brunori et al., 1973), are 70% and 74% respectively.

Electrophoretic and Spectral Properties of Reconstituted Hb's. A great number of different preparations were titrated with the three hemes. It was found that only 70–80% of the globin is titratable, and, as previously reported (Fioretti et al., 1976), a slightly higher value was always obtained with protoheme, indicating a better reconstitution of the apoprotein with the natural heme. For additional investigations both globins were reconstituted with the stoichiometric amounts of proto-, meso-, and deuterohemes as calculated from the respective titrations.

All the reconstituted proteins are electrophoretically homogeneous and have the same anionic mobility as the corresponding proto-native Hb's. The ferric derivative obtained after reconstitution can be converted, with the procedure reported above, into the deoxy, oxy, and carbonmonoxy derivatives. The formation of a stable oxygenated species obtained in all cases indicates that nonspecific heme–globin association products are practically absent.

The absorption properties of the ferrous derivatives for both proteins reconstituted with proto-, meso-, and deuteroheme are reported in Table I. The optical properties of the proto reconstituted proteins are very similar to those of the native proteins. As previously reported for human hemoglobin (Antonini et al., 1964), also for Hb I and Hb IV a blue shift of the absorption maxima is observed going from proto- to meso- to deutero-Hb. This has been correlated with the decreased electron-withdrawing power of the two substituents of the porphyrin ring in positions 2 and 4 (Caughey et al., 1966). Comparison of the λ_{max} of the α band of proto-, meso-, and deutero-HbO_2A (Antonini et al., 1964) with the same derivatives of trout hemoglobins shows consistently that the blue shift is more marked for the Hb's from trout as compared with HbA. This suggests that in the former cases the electron-withdrawing capacity of the 2,4 substituents is further decreased.

Functional Properties of Reconstituted Hb's I and IV. (a) Oxygen and Carbon Monoxide Equilibria. Hb trout I and IV reconstituted with protoheme yield products whose functional behavior is very similar, though not identical, to that of the native hemoglobins. Thus, the reconstituted proto-Hb's show

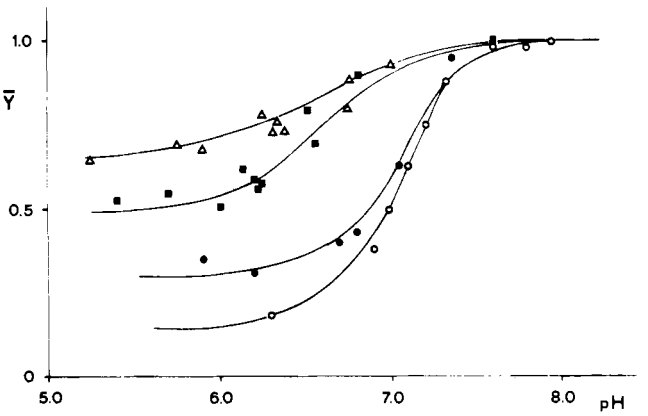

FIGURE 1: Root effect of native (○) and reconstituted Hb trout IV with proto- (●), meso- (■), and deutero- (△) heme; the experiments were performed in phosphate buffer (0.2 M), containing 10^{-3} M IHP, at 10 °C.

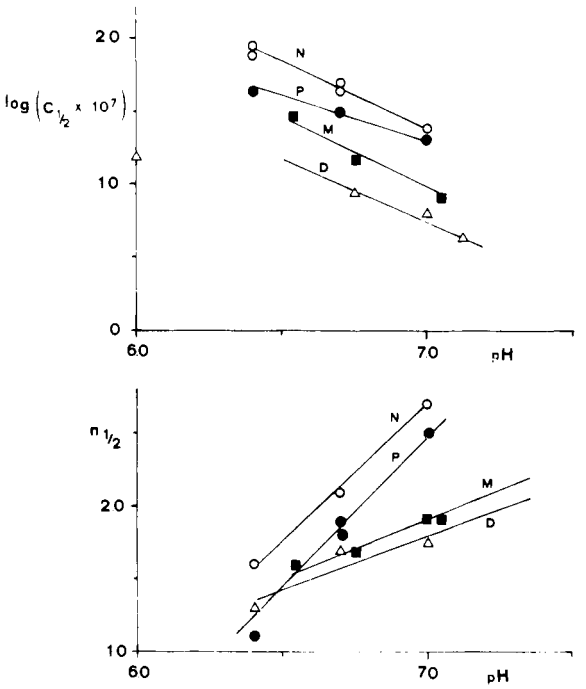

FIGURE 2: pH dependence of the CO equilibria of native (○) and reconstituted Hb trout IV with proto- (●), meso- (■), and deutero- (△) heme. The experiments were carried out at 10 °C in phosphate buffer (0.1 M).

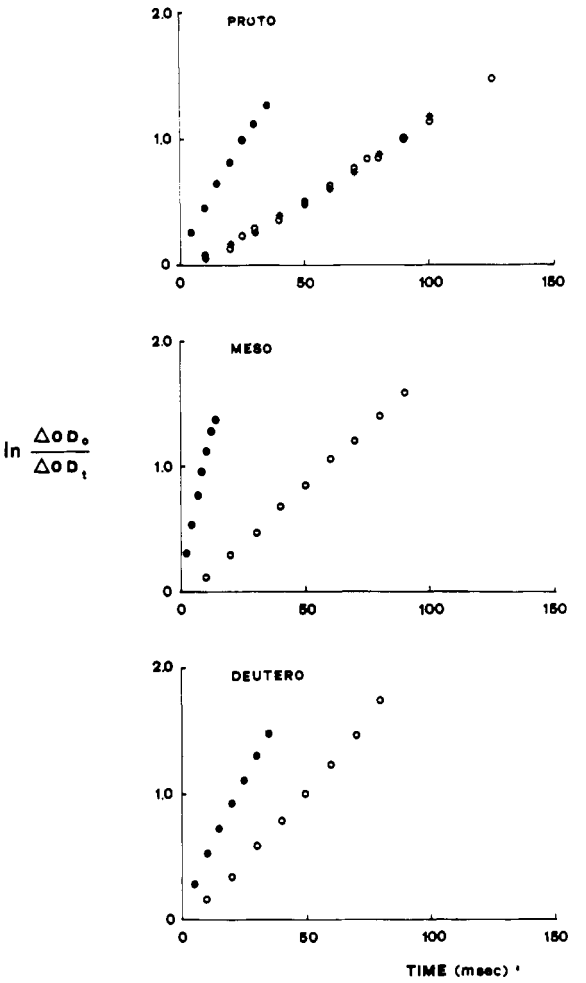

FIGURE 3: CO binding kinetics of Hb I reconstituted with proto-, meso-, and deuteroheme. Experiments were carried out at 20 °C in phosphate buffer (0.1 M) at pH 7.0 under conditions of partial (closed symbols) and total (open symbols) photodissociation. CO concentration = 100 μM; Hb concentration = 3–6 μM (heme); observation wavelength = 431 nm for proto- and 420 nm for meso- and deutero-Hb's. Partial photodissociation corresponds to 30–35% breakdown of the CO complex. Asterisks refer to the behavior of the native protein under the same conditions.

slight differences in ligand affinity and cooperativity, an increase of the former paralleling a decrease of the latter.

The oxygen affinity ($p_{1/2}$) and the values of $n_{1/2}$ obtained from meso and proto reconstituted Hb's I and IV at pH above 7 are reported in Table II. Because of the instability of the material during deoxygenation, no reliable O_2 binding data could be obtained for deutero-Hb IV and I.

In the case of Hb IV the characteristic functional behavior is maintained in the reconstituted proteins, as shown by the marked pH dependence of both ligand affinity and cooperativity (see below). The pH dependence of the fractional saturation with oxygen at constant O_2 pressure (~155 mmHg) is reported in Figure 1 for different preparations of the purified proto-, meso-, and deutero-Hb's IV. Between native and proto-reconstituted Hb IV only a small difference at pH ≤6.5 is observed, while at each pH value the fractional saturation with oxygen is much higher for both meso- and deutero-Hb's IV. It may be concluded, therefore, that the Root effect shows considerable differences between proto- (native or reconstituted) Hb IV, on one hand, and deutero- or meso-reconstituted Hb IV on the other.[1] Substitution of protoheme respectively with meso- and deuteroheme causes, at each pH value, an increase of carbon monoxide affinity which is always higher for the deutero reconstituted protein (Figure 2). Although lower values of $n_{1/2}$ and higher affinities are obtained for the proto-reconstituted Hb as compared with the native protein, the general behavior of ligand binding is maintained. Thus, the Bohr effect, as well as the characteristic pH dependence of the heme–heme interactions, are similar in extent for both proteins (Figure 2).

For meso- and deutero-Hb IV the Bohr effect, in spite of the higher overall affinity, is similar in amplitude to that observed for the native protein, at least in the pH range examined. In both cases, heme–heme interactions are considerably decreased but still present and significantly pH dependent, the value of

[1] It was previously found (Fioretti et al., 1976) that meso-Hb IV at pH around 6 in air had an average saturation equal, or may be slightly higher, than that of deutero-Hb IV, while the data shown in Figure 1 indicate the opposite behavior. We believe that this difference may be attributed to a variability in the properties of the reconstituted hemoglobins obtained with earlier, nonpurified, preparations of globin.

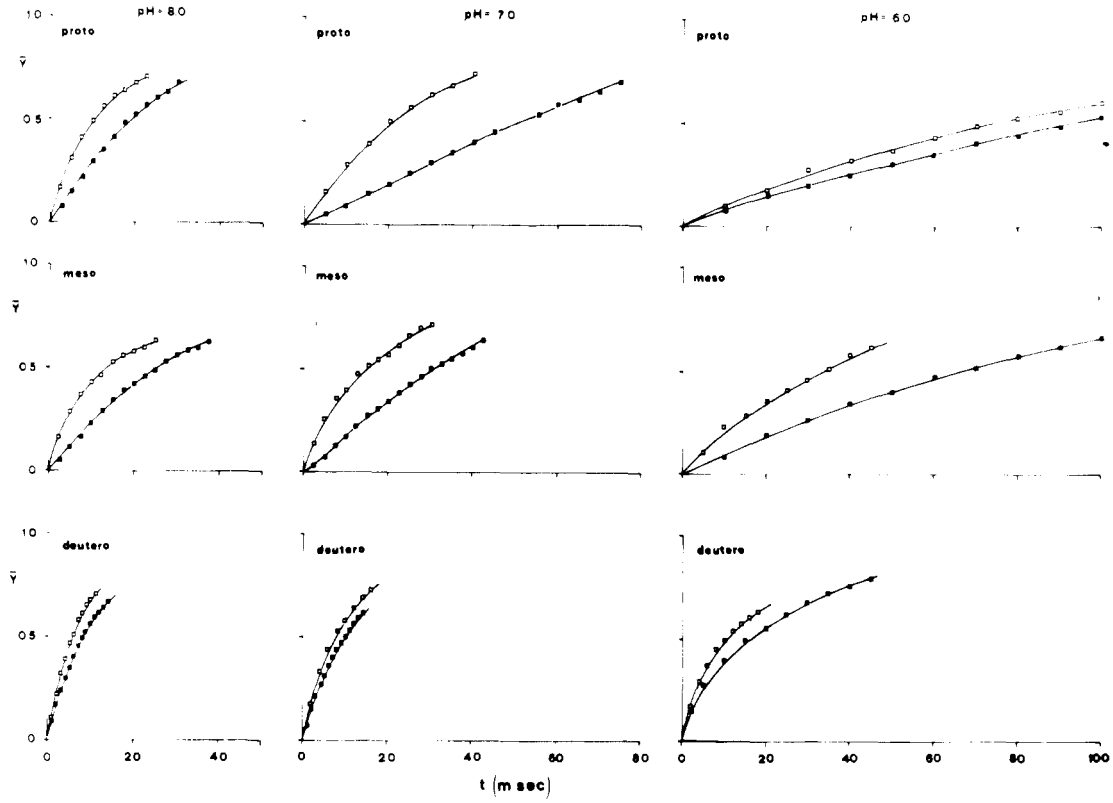

FIGURE 4: CO binding kinetics of Hb IV reconstituted with proto-, meso-, and deuteroheme. Experiments were carried out at 20 °C in phosphate buffer (0.1 M) at pH 6–8 under conditions of partial (open symbols) and total (closed symbols) photodissociation. CO concentration = 200 μM; Hb concentration = 2–3 μM (heme); observation wavelength = 431 nm for proto- and 416 nm for meso- and deutero-Hb's. Partial photodissociation corresponds to 30–35% breakdown of the CO complex.

$n_{1/2}$ going from 1.8 to 1.3 as the pH is decreased from ~7 to ~6.5 (Figure 2).

(b) Kinetics of Carbon Monoxide Binding. The combination of the reconstituted Hb's I with carbon monoxide after complete photodissociation is shown in Figure 3. The kinetic behavior of proto-reconstituted Hb I is identical with that of the native protein, and the autocatalytic character of the progress curve is evident also for meso- and deutero-reconstituted Hb I. The general trend, at constant pH, points to an increase in the overall association rate constant and to a decrease in the autocatalytic character in the order proto- > meso- > deutero-Hb I. The effect of partial photodissociation is clearly discernible and very similar for proto- and meso-reconstituted Hb's I, but obviously reduced in the case of deutero-Hb I. This is in agreement with the decrease in the autocatalytic character of the progress curve and, together with the higher value of the apparent association rate constant, points to a substantial stabilization of a high affinity state of the molecule.

The results of flash photolysis experiments on the CO binding kinetics of Hb IV are reported in Figure 4. The kinetic behavior is consistent with the equilibrium data described above. Thus, similarly to what was found for the native protein (Giardina et al., 1973), the time course of CO binding by reconstituted proto-Hb IV is clearly autocatalytic at alkaline pH values and tends to become approximately pseudo-first-order going to acid pH values (~6.0). An autocatalytic time course is also observed, contrary to the previous results on human and horse hemoglobins (Antonini & Gibson, 1960; Seybert et al., 1976), for meso-Hb IV; in this case, however, the pH dependence of both the shape and the half-time of the process is less pronounced. The kinetic behavior of deutero-Hb IV is, on the other hand, nearly pH independent and the time course of the reaction is very closely pseudo-first-order.

The results of partial photodissociation experiments on Hb IV are also in qualitative accord with the equilibrium data, based on an interpretation within the framework of a simple two-state model (Hopfield et al., 1971; Monod et al., 1965). A current interpretation of the Root effect in Hb trout IV (Brunori, 1975; Giardina et al., 1975) is based on a progressive stabilization by protons (and organic phosphates) of a low affinity quaternary state of the molecule, which at low pH is the only state significantly populated at every degree of ligation. For native Hb IV as well as proto-reconstituted Hb IV, the kinetics of CO combination upon partial photolysis is consistent with this hypothesis and with similar data available on hemoglobin from carp (Tan et al., 1973).

The results on deutero-Hb IV (Figure 4) show that the effect of partial photolysis is practically absent at every pH value, in full agreement with the idea of a relative stabilization of a high affinity state of the molecule at every pH value. Meso-reconstituted Hb IV, on the other hand, seems to represent an intermediate case.

Thus at pH ~6 the results for the three reconstituted hemoglobins suggest, qualitatively, the following interpretation: (i) proto-Hb IV is (almost) completely in a low-affinity state and therefore full and partial photodissociations yield (almost) the same CO combination time course, characterized by a low combination velocity constant ($l' = 0.34 \times 10^5$ M^{-1} s^{-1}); (ii) in deutero-Hb IV a high-affinity state is prevailing, and this is indicated by the low value of n, the (relatively) high CO affinity, and the lack of effect of partial photodissociation on the CO combination kinetics; the overall combination rate constant is significantly higher ($l' = 1.48 \times 10^5$ M^{-1} s^{-1}); (iii) meso-Hb IV corresponds to an intermediate condition; the overall combination rate constant at pH 6.0 is intermediate ($l' = 0.57 \times 10^5$ M^{-1} s^{-1}) and the effect of partial photodisso-

ciation is observed clearly at all pH values.

For Hb IV the kinetic experiments with carbon monoxide show that the effect of pH on the apparent association rate constant is still evident for meso-Hb and very much smaller for deutero-Hb IV. This fact, taken together with the similarity in the Bohr effect observed in the carbon monoxide binding experiments (Figure 2), implies a substantial contribution of the kinetic dissociation rate constant(s) to the Root effect. Furthermore the large pH dependence of carbon monoxide affinity still observed in the case of deutero-Hb IV, for which the effect of partial photodissociation is practically absent and cooperativity drastically reduced, points to the existence of a different control mechanism for heme–heme interactions and ligand affinity. Therefore discrete conformational events seem to be involved in homo- and heterotropic interaction effects in trout hemoglobin.

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