Dietrich, R. F., & Kenyon, G. L. (1978) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 37*, 1804.
Dietrich, R. F., Sakurai, T., & Kenyon, G. L. (1979) *J. Org. Chem. 44*, 1894.
Dietrich, R. F., Marletta, M. A., & Kenyon, G. L. (1980) *Org. Magn. Reson. 13*, 79.
Kenyon, G. L., & Fee, J. A. (1973) *Prog. Phys. Org. Chem. 10*, 381–410.
Kenyon, G. L., Struve, G. E., Kollman, P. A., & Moder, T. I. (1976) *J. Am. Chem. Soc. 98*, 3695.
Kuby, S. A., Noda, L., & Lardy, H. A. (1954) *J. Biol. Chem. 209*, 191.
Maggio, E. T., Kenyon, G. L., Markham, G. D., & Reed, G. H. (1977) *J. Biol. Chem. 252*, 1202.
Mahowald, T. A., Noltmann, E. A., & Kuby, S. A. (1962) *J. Biol. Chem. 237*, 1535.
McLaughlin, A. C., Cohn, M., & Kenyon, G. L. (1972) *J. Biol. Chem. 247*, 4382.
McLaughlin, A. C., Leigh, J. S., Jr., & Cohn, M. (1976) *J. Biol. Chem. 251*, 2777–2787.
Meirovitch, E., & Poupko, R. (1978) *J. Phys. Chem. 82*, 1920–1925.
Mildvan, A. S., & Cohn, M. (1970) *Adv. Enzymol. 33*, 1–70.
Morrison, J. F., & James, E. (1965) *Biochem. J. 97*, 37.
Morrison, J. F., & Cleland, W. W. (1966) *J. Biol. Chem. 241*, 673.
Noda, L., Kuby, S. A., & Lardy, H. A. (1954) *J. Biol. Chem. 239*, 203.
Quitt, P., Hellerbach, J., & Vogler, K. (1963) *Helv. Chim. Acta 46*, 327.
Reed, G. H., & Cohn, M. (1972) *J. Biol. Chem. 247*, 3073.
Reed, G. H., & McLaughlin, A. C. (1973) *Ann. N.Y. Acad. Sci. 222*, 118–129.
Reed, G. H., Cohn, M., & O'Sullivan, W. J. (1970) *J. Biol. Chem. 245*, 6547–6552.
Reed, G. H., Barlow, C. H., & Burns, R. A., Jr. (1978) *J. Biol. Chem. 245*, 6547–6552.
Rodebaugh, R. M., & Cromwell, N. H. (1969) *J. Heterocycl. Chem. 6*, 993.
Rowley, G. L., & Kenyon, G. L. (1974) *Anal. Biochem. 58*, 525.
Rowley, G. L., Greenleaf, A. L., & Kenyon, G. L. (1971) *J. Am. Chem. Soc. 93*, 5542.
Yue, R. H., Palmieri, R. H., Olson, O. E., & Kuby, S. A. (1967) *Biochemistry 6*, 3204–3227.

Mechanism of Action of Plasma Amine Oxidase Products Released under Anaerobic Conditions†

Kenneth A. Berg and Robert H. Abeles*

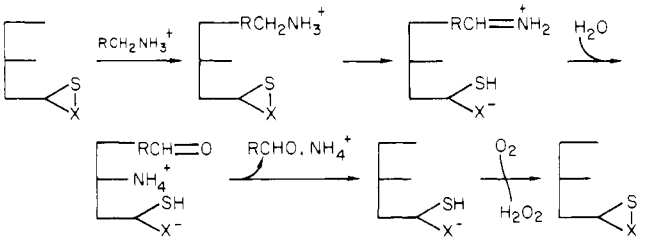
ABSTRACT: A mechanism that we have proposed for bovine plasma amine oxidase predicts that under anaerobic conditions (single turnover) 1 mol of benzaldehyde and 1 mol of NH_4^+ are produced from benzylamine. Other works have reported experiments with amine oxidases from other sources that show that NH_4^+ is not released under anaerobic conditions. The amount of NH_4^+ and benzaldehyde released when plasma amine oxidase acts on benzylamine under anaerobic conditions has been determined to resolve this discrepancy. It was found that 1 mol of NH_4^+ and 1 mol of benzaldehyde are released per active site. This result is consistent with the mechanism that we have proposed but is inconsistent with other mechanisms that invoke pyridoxal as an active-site component of amine oxidase.

Bovine plasma amine oxidase, a Cu^{2+}-containing protein, catalyzes the oxidation of primary amines (eq 1). Similar

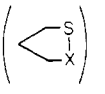
$$RCH_2NH_3^+ + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_4^+ \quad (1)$$

enzymes have also been isolated from other sources. It is generally agreed that the reaction is a two-step process involving, first, the reduction of the enzyme by the substrate and then the oxidation of the reduced enzyme by O_2. There is, however, considerable disagreement concerning the immediate product that results from the oxidation of the substrate and the nature of the group on the enzyme that becomes reduced. We have proposed a mechanism for plasma amine oxidase, as shown in Scheme I (Suva & Abeles, 1978). The active site

Scheme I



of the enzyme contains an as yet incompletely identified structure

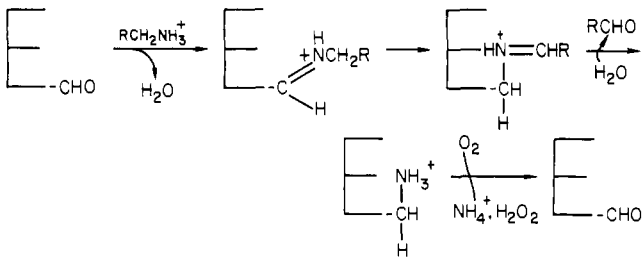


which can function as an electron acceptor. The sulfur component of this structure is provided by cysteine, which is bonded to an unidentified atom. Reaction of the amine substrate with enzyme results in the reduction of the prosthetic group and formation of an imine. The imine is hydrolyzed on the enzyme

† Publication No. 1309 from the Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254. *Received February 6, 1980.* This research was supported in part by a grant from the National Institutes of Health (GM 12633) to R.H.A. and by National Institutes of Health Training Grant GM 00212 and Gustave and Hattie Klein Fellowship to K.A.B.

0006-2960/80/0419-3186$01.00/0 © 1980 American Chemical Society

Scheme II[a]



[a] CHO is the aldehyde group of enzyme-bound pyridoxal.

so that the actual products released from the enzyme are NH_4^+ and aldehyde. It has been shown that under anaerobic conditions aldehyde is released stoichiometric with enzyme (Oi et al., 1970; Reed & Swindell, 1969).

Several reports have appeared in the literature that are inconsistent with the mechanism that we have proposed. Experiments with pig plasma amine oxidase led to the conclusion that, under anaerobic conditions, benzaldehyde is released (Lindström et al., 1974; Lindström & Petterson, 1978a) from benzylamine but not NH_4^+ (Lindström & Petterson, 1978b). Similar results were also reported for amine oxidase from *Aspergillus niger* (Suzuki et al., 1971). These studies were done with *n*-butylamine, and it was concluded that butyraldehyde, but not NH_4^+, is released under anaerobic conditions. A different permutation of results was obtained in another study with pig plasma amine oxidase (Taylor et al., 1972). These authors concluded that, under anaerobic conditions, NH_4^+, but not benzaldehyde, is released from benzylamine. This work has been criticized on the grounds that appropriate corrections were not made for NH_4^+ contamination in the benzylamine added (Lindström & Petterson, 1978b).

The failure to observe NH_4^+ release is difficult to reconcile with the mechanism (Scheme I) that we have proposed. That mechanism requires that under anaerobic conditions, i.e., single turnover conditions, 1 mol of NH_4^+ and 1 mol of aldehyde should be released per active site. The authors cited above favor a mechanism in which pyridoxal or a structurally similar entity participates in the reaction. A mechanism involving pyridoxal is shown in Scheme II (Blashko, 1962). According to that mechanism, a transamination takes place between the amine substrate and pyridoxal, which results in the formation of aldehyde and pyridoxamine. Pyridoxamine is then oxidized to pyridoxal and NH_4^+. The mechanism requires that aldehyde but not NH_4^+ be released under anaerobic conditions. It should be noted that Taylor et al. (1972) also favor the pyridoxal mechanism, although they observed NH_4^+ release under anaerobic conditions. In order to reconcile the pyridoxal mechanism with their results, an unusual reaction was invoked, which to the best of our knowledge has no chemical precedence.

The available data concerning NH_4^+ and aldehyde release under anaerobic conditions are confusing and contradictory. Failure to release NH_4^+ under anaerobic conditions would throw serious doubts on the mechanism that we have proposed. On the other hand, the demonstration that NH_4^+ and aldehyde are released under anaerobic conditions would provide a strong argument against the pyridoxal mechanism. We have, therefore, undertaken a series of experiments with bovine plasma amine oxidase in which we determined the amount of NH_4^+ released under anaerobic conditions from benzylamine. These determinations present experimental difficulties, since background levels of NH_4^+ are generally high. We have devised procedures to minimize this difficulty. Furthermore, we have measured NH_4^+ release at several enzyme levels, since this provides an internal check on the accuracy of the measurements. We have also measured benzaldehyde release under conditions similar to those under which NH_4^+ release was measured.

Materials and Methods

Enzyme Purification and Assay. Bovine plasma amine oxidase was isolated by the method of Yamada & Yasunobu (1962) and stored as the ammonium sulfate pellet following the DEAE-Sephadex column. Immediately before use, a portion of the pellet was dialyzed against 50 mM potassium phosphate buffer, pH 7.5, and was chromatographed on an aminohexyl-Sepharose affinity column (Toraya et al., 1976). The enzyme was precipitated with ammonium sulfate and dialyzed against the buffer used in the release experiment. Enzyme activity was determined by measuring the rate of formation of *p*-hydroxybenzaldehyde from *p*-hydroxybenzylamine under the following conditions: 40 μM *p*-hydroxybenzylamine·HCl, enzyme, and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.0 mL, 25 °C. The change in optical density at 330 nm was recorded. For *p*-hydroxybenzaldehyde, $\epsilon_{330nm} = 25\,000\ M^{-1}\ cm^{-1}$. One spectral unit (SU) is defined as 1×10^{-3} ΔOD/min. Pure enzyme has a specific activity of 9000 SU/mg or 0.36 IU/mg (Suva, 1978). The enzyme molecular weight is 85 000 per monomer. Under assay conditions, 1 nmol of monomer corresponds to 765 SU.

Preparation of Enzyme and Substrate for NH_4^+ Release Experiments. Three milliliters of enzyme (approximately 5 mg/mL) was dialyzed against 500 mL of 100 mM potassium phosphate buffer containing 20 g of activated carbon (Darco G-60, Fisher Scientific Co.). Before use, the activated carbon was washed with deionized water. All buffers were prepared with doubly distilled water. The second distillation was carried out with a glass still. Buffers were treated with activated carbon (~20 g/L) prior to use. It was found that NADH (Sigma) contained significant amounts of NH_4^+. To remove NH_4^+, a solution of 0.2 mg of NADH in 0.5 mL of 3 mM potassium phosphate buffer, pH 7.6, was placed on a Bio-Gel P-2 column (1.1 × 22 cm) and eluted with 3 mM potassium phosphate buffer, pH 7.6. The column eluant containing NADH was lyophilized and redissolved before use in 100 mM potassium phosphate buffer, pH 7.6. Benzylamine·HCl was recrystallized from ethanol. It was found to be essentially free of NH_4^+ when assayed with glutamate dehydrogenase.

A mixture of glucose oxidase, glutamate dehydrogenase, and catalase, used in the NH_4^+ experiment, was dialyzed against 100 mM potassium phosphate buffer, pH 7.6, containing activated carbon.

Enzymes and Cofactors. All enzymes and cofactors were obtained from Sigma Chemical Co.

Anaerobic Cell. An anaerobic spectrophotometer cell was built by gluing a brass fitting to the top of a 1-mL quartz cuvette. A round 10 × 4 mm Hewlett-Packard gas chromatography septum was seated into the fitting. A threaded brass cap with a 5-mm hole in the center squeezes the septum into the fitting, providing a tight seal, but allowing the passage of hypodermic needles. A small magnetic stirring bar was placed into the cell. This was used to stir the solution with an external magnet.

Results

NH_4^+ Release from Benzylamine under Anaerobic Conditions. In this experiment, as well as other anaerobic ex-

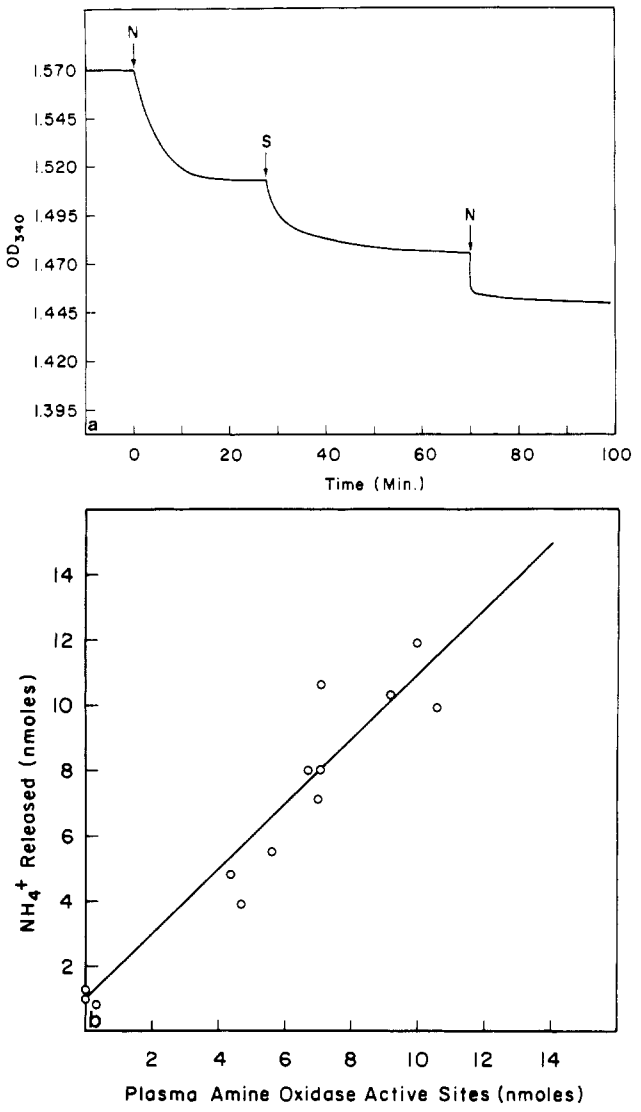


FIGURE 1: (a) NH_4^+ release by plasma amine oxidase from benzylamine under anaerobic conditions. The anaerobic cuvette contained plasma amine oxidase, 7.1 nmol (active sites), 0.1 M D-glucose, and 22 mM α-ketoglutarate in 100 mM potassium phosphate buffer, pH 7.6, total volume 0.90 mL. The cell was sealed and flushed with argon for 4 min. A solution (0.1 mL) of glutamate dehydrogenase (200 mIU), glucose oxidase (600 mIU), and catalase (200 mIU) in 0.1 M potassium phosphate buffer, pH 7.6, was injected through the septum. After 20 min, 160 nmol of NADH in 4 μL was injected. The reaction was allowed to proceed (about 30 min) until no more NADH was consumed. At this point (N) approximately 10 nmol of an O_2-free solution of NH_4Cl in 20 μL was added to determine whether the assay system was functional. A saturated solution of benzylamine·HCl (2 μL; ~4 μmol) was then injected (S), and the reaction was allowed to proceed until no more NADH was consumed. At that point (N), NH_4Cl was again injected to test whether the assay system was functional. (b) Stoichiometry of NH_4^+ release. Experimental conditions and procedures are as in a, except plasma amine oxidase was varied. For 0 nmol of plasma amine oxidase active site, enzyme was omitted and replaced with buffer.

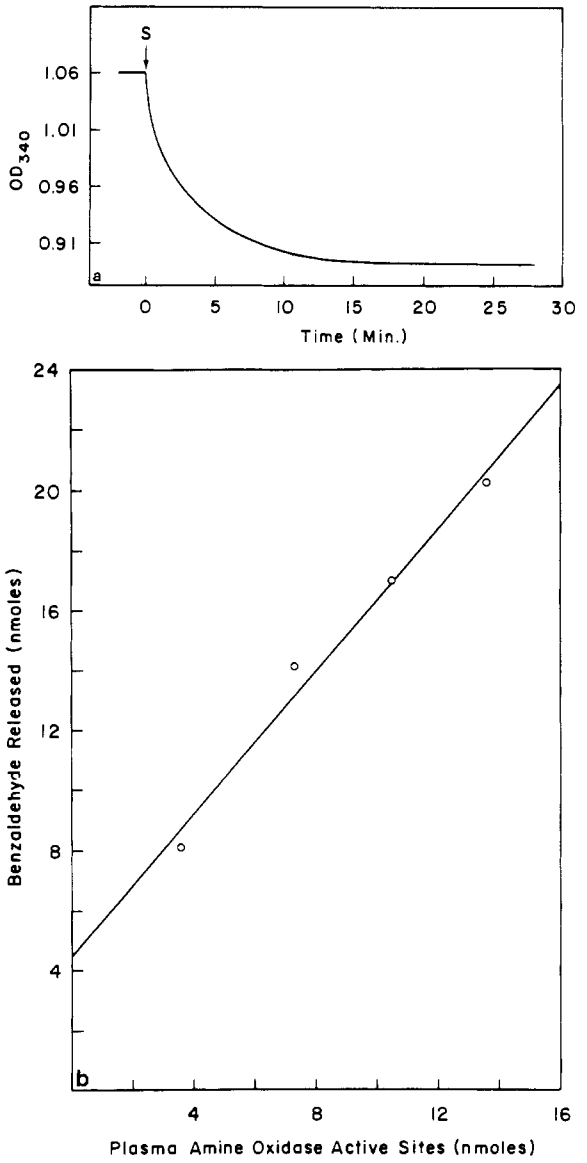


FIGURE 2: (a) Benzaldehyde release under anaerobic conditions. The anaerobic cuvette contained plasma amine oxidase (22.6 nmol) (active sites), glucose (0.1 M), liver alcohol dehydrogenase (40 mIU), catalase (200 mIU), and NADH (0.15 mM), in 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 1.0 mL. The cuvette was sealed and flushed with argon for 4 min, and 600 mIU of glucose oxidase in 5 μL was injected. The cuvette was maintained at 25 °C with occasional stirring. Benzylamine·HCl (~4 μmol in 2 μL) was injected (S). (b) Stoichiometry of benzaldehyde release. Experimental conditions are as in a, except the plasma amine oxidase concentration was varied.

periments, anaerobic conditions were obtained by the addition of glucose, glucose oxidase, and catalase to the reaction mixture. This allowed us to achieve an oxygen concentration of less than 0.1 μM. It was established in separate experiments that neither glucose nor glucose oxidase interferes with the action of plasma amine oxidase. NH_4^+ release was determined from the amount of NADH consumed in the presence of glutamate dehydrogenase and α-ketoglutarate. The experimental details are given in the legend to Figure 1a. Figure 1a shows the time course of NH_4^+ release from benzylamine under anaerobic conditions. Figure 1b shows the amount of NH_4^+ formed per active site for several experiments in which the total amount of plasma amine oxidase was varied. The number of nanomoles of NH_4^+ liberated was determined from the slope of the line in Figure 1b; 1.02 ± 0.11 nmol of NH_4^+ is released per nmol of active site.

Benzaldehyde Release from Benzylamine under Anaerobic Conditions. Benzylamine was added to plasma amine oxidase under anaerobic conditions, and the amount of benzaldehyde released was determined from the amount of NADH consumed in the presence of liver alcohol dehydrogenase. Figure 2a shows the time course of benzaldehyde release. In Figure 2b, the amount of benzaldehyde released at various enzyme concentrations is shown. From the slope of the line in Figure 2b, it was determined that 1.19 ± 0.07 nmol of benzaldehyde is released per nmol of active site. This is in agreement with

previously reported results (Oi et al., 1970; Reed & Swindell, 1969).

Discussion

The results reported here show that addition of benzylamine to plasma amine oxidase under anaerobic conditions results in the formation of 1 mol of benzaldehyde and 1 mol of NH_4^+ per mol of enzyme subunit (active site). These results are consistent with the mechanism that we have proposed involving the oxidation of the substrate to an enzyme-bound Schiff base, which is hydrolyzed prior to release from the enzyme (Scheme I). On the other hand, the results reported here do not agree with the proposal that the immediate product of the oxidation is the aldehyde and pyridoxamine. Therefore, these results together with previous experiments (Suva & Abeles, 1978) make the pyridoxal mechanism for bovine plasma amine oxidase untenable. We wish to point out that the reports, referred to above, that NH_4^+ is not released under anaerobic conditions were based on experiments with enzymes other than the beef plasma enzyme. It is, therefore, possible that amine oxidase from other sources operates through a different mechanism. In view of the many similar properties which these amine oxidases show, we consider that possibility unlikely. We believe that none of the amine oxidases is a pyridoxal enzyme.

References

Blashko, H. (1962) *Biochemistry 1*, 68.

Lindström, A., & Petterson, G. (1978a) *Eur. J. Biochem. 83*, 131.

Lindström, A., & Petterson, G. (1978b) *Eur. J. Biochem. 84*, 479.

Lindström, A., Olsson, B., & Petterson, G. (1974) *Eur. J. Biochem. 42*, 377.

Oi, S., Inamasu, M., & Yasunobu, K. T. (1970) *Biochemistry 9*, 3378.

Reed, D. J., & Swindell, R. (1969) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 28*, 891.

Suva, R. H. (1978) Ph.D. Thesis, Brandeis University, Waltham, MA.

Suva, R. H., & Abeles, R. H. (1978) *Biochemistry 17*, 3538.

Suzuki, H., Ogura, Y., & Yamada, H. (1971) *J. Biochem. 69*, 1065.

Taylor, C. E., Taylor, R. S., Rasmussen, C., & Knowles, P. F. (1972) *Biochem. J. 130*, 713.

Toraya, T., Ujimura, J., Ikeda, S., Fukui, S., Yamada, H., & Kumagai, H. (1976) *Biochim. Biophys. Acta 420*, 316.

Yamada, H., & Yasunobu, K. T. (1962) *J. Biol. Chem. 237*, 1511.

Role of C-Terminal Histidine in the Alkaline Bohr Effect of Human Hemoglobin†

John V. Kilmartin, Jan H. Fogg, and Max F. Perutz*

ABSTRACT: We have determined the alkaline Bohr effect in concentrated hemoglobin solution (~100 mg/mL) at low ionic strength (10 mM Cl^-) in the absence of phosphate in two chemically modified hemoglobins (desHis-146β hemoglobin and hemoglobin *N*-ethylsuccinimide) in which only the contribution of the C-terminal histidine to the alkaline Bohr effect is inhibited. We found that either of these modifications results in the inhibition of 60% of the alkaline Bohr effect. We have also compared the structure of salt-free methemoglobin crystals with that of methemoglobin crystals precipitated from ammonium sulfate. A difference electron-density map shows the salt bridge between the C-terminal histidine and the nearby aspartate FG1(94)β to be absent in both. These results disprove the conclusions of Ho & Russu [Ho, C., & Russu, I. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., Ed.) pp 179–194, Academic Press, New York], Russu et al. [Russu, I. M., Ho, N. T., & Ho, C. (1980) *Biochemistry 19*, 1043–1052], and Matthew et al. [Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 1928–1936] that in the absence of phosphate and at low concentrations of chloride the salt bridge remains intact in liganded hemoglobins and that the C-terminal histidine therefore makes only a minor contribution to the alkaline Bohr effect. On the contrary, our results show that the fractional contribution of the histidine increases at low chloride concentration. The p*K* changes calculated by Matthew et al. [Matthew, J. B., Hanania, G. I. H., & Gurd, F. R. N. (1979) *Biochemistry 18*, 1919–1928] are based on an incorrect position of this residue in oxyhemoglobin; when this residue is positioned correctly, the equations of Matthew and co-workers predict the major p*K* change which we have observed.

"Love people, slay errors."—St. Augustine.

One of the successes of the X-ray analysis of hemoglobin was a structural interpretation of most of the alkaline Bohr effect (Perutz et al., 1969; Kilmartin & Rossi-Bernardi, 1969; O'Donnell et al., 1980). In deoxyhemoglobin, which has the quaternary T structure, the terminal residues form the salt bridges shown in Figure 1, whereas in liganded hemoglobins, which have the R structure, they are free. In the light of this evidence we suggested that the pK_a values of the C-terminal histidine [HC3(146)β] and the N-terminal valine [NA1(1)α] are normal in liganded hemoglobins but are raised through their linkage to anionic groups in deoxyhemoglobin. This was confirmed by chemical studies which showed that the histidine contributes ~40% and the valine 20–30% to the alkaline Bohr effect (Kilmartin, 1977). The contribution of the histidine was

† From the MRC Laboratory of Molecular Biology, University Postgraduate Medical School, Cambridge CB2 2QH, England. *Received November 19, 1979.*

0006-2960/80/0419-3189$01.00/0 © 1980 American Chemical Society